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# Mixotrophic flagellates in coastal marine sediments: quantitative role and ecological significance

by
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# Contents

# Chapter 1

## General Introduction

Planktonic protists have traditionally been divided into phototrophic phytoplankton and phagotrophic zooplankton due to the presence or absence of cellular plastids, although the arbitrary nature of this division has become increasingly obvious (Corliss 1986). The existence of mixotrophs, which are able to combine autotrophic and heterotrophic modes of nutrition, has already been described in the first half of last century (Pascher 1917, Biecheler 1936). Only recently, though, there has been an increasing interest in the quantitative and qualitative role of mixotrophic protists in plankton communities (see reviews by Porter 1988, Sanders & Porter 1988, Sanders 1991, Jones 1994, Holen & Boraas 1995, Sanders et al. 1989) due to the increasing recognition of the 'microbial loop' as important pathway for carbon and nutrient flux in aquatic ecosystems (Azam et al. 1983). Acting on more than one trophic level, mixotrophs have complicated original models for the microbial loop.

In a broader definition of mixotrophy, phagotrophy is only one of several possible forms of heterotrophy, besides osmotrophy, which is defined as absorbing organic compounds in soluble form, either by transport across the plasma membrane or by pinocytosis (Jones 1994). In the ecological literature, though, the term mixotrophy is primarily used for protists that are both phototrophic and phagotrophic (Dolan 1992, Holen & Boraas 1995, Jones 1997, Jones 1994, Reimann et al. 1995, Sanders 1991, Stoecker 1991).

Mixotrophy in this restricted sense has been observed in a number of planktonic protists, including phytoflagellates, ciliates and sarcodines, and has been recorded in eutrophic, mesotrophic, and oligotrophic waters ranging from freshwater ponds to the open ocean (Sanders 1991, Riemann et al. 1995, Stoecker 1998).

Mixotrophs include both phagotrophic algae that are primarily phototrophic (Green 1991, Holen & Boraas 1995, Raven 1997, Schnepf & Elbrächter 1992) and photosynthetic protozoa that are primarily phagotrophic. In many cases, like in ciliates, freshwater heliozoa or benthic marine foraminifera, photosynthetic protozoa are photosynthetic due to the presence of algal endosymbionts or due to sequestering and utilizing ingested chloroplasts (Anderson 1993, Beaver & Crisman 1989, Rogerson et al. 1989, Patterson & Dürrschmidt 1987, Caron & Swanberg 1990, Dolan 1992, Jones 1994, Laval-Peuto 1992, Reisser 1992 and Stoecker et al. 1996).

Mixotrophic phytoflagellates include chrysophytes, dinoflagellates, prymnesiophytes and cryptophytes. Numerous planktonic studies described their occurrence, their physiological

peculiarities, their contribution to nutrient recycling and their function within microbial food webs (Bird & Kalff 1987; Bennett et al. 1988; Caron et al. 1990; Sanders et al. 1990; Stoecker et al. 1997, Hall et al. 1993, Havskum & Hansen 1997, Havskum & Riemann 1996, Kimura & Ishida 1989, Sanders et al. 1989). It was shown that mixotrophic occurrence and abundances in marine and freshwater ecosystems are highly variable in temporal and spatial scales, but that mixotrophs can play a major role as primary producers and as phagotrophs. Mixotrophs were found to contribute up to 50% to the total phototrophic nanoplankton in different marine and freshwater habitats (e.g. Arenovski et al. 1995, Sanders et al. 2000, Havskum & Riemann 1996, Berninger et al. 1992). As phagotrophs, they contributed up to 60% of the bacterivory and 57% of the herbivory on picophytoplankton and small (<5μm) nanophytoplankton, also varying in space and time (e.g. Berninger et al. 1992, Hall et al. 1993, Nygaard & Tobiesen 1993, Havskum & Riemann 1996, Havskum & Hansen 1997, Jansson et al. 1996, Safi & Hall 1999). These sometimes large proportions of MNF have important implications for algal nutrition, nutrient dynamics and food web interactions in planktonic ecosystems.

Potential ecological advantages of phagotrophy for phytoflagellates still remain largely speculative (Sanders et al. 2001), but include support of growth in the dark, supplementation of photosynthetic carbon fixation and acquisition of macronutrients (nitrogen and phosphorus) and micronutrients (e.g. acquisition of vitamins, essential fatty acids, iron) (Jones 1994, Keller et al. 1994, Nygaard & Tobiesen 1993, Sanders 1991, Maranger et al. 1998). Phagotrophy is likely to fulfill different requirements for different phytoplankton species along a mixotrophy gradient ranging from nearly pure phototrophy to nearly pure heterotrophy (Jones 1994, Sanders et al. 1990). In addition, changes of environmental parameters such as light level, nutrient concentrations, prey abundances etc., may cause shifts within a species in the relative importance of photosynthesis and phagotrophy, or in the specific role that phagotrophy performs (Keller et al. 1994, Nygaard & Tobiesen 1993, Urabe et al. 1999, Urabe et al. 2000).

Acting both as primary producers and as consumers of particulate organic matter, mixotrophic protists may play a pivotal role in aquatic microbial food webs. However, the gradient among mixotrophs with some species more autotrophic and some species more heterotrophic indicates that mixotrophy is regulated in different ways in different protists and can play different roles in food web dynamics (Jones 1997, Jones 1994). Diverse functional types of mixotrophy were proposed to affect the total productivity of the microbial food web, with phagotrophy by algae decreasing the total microbial food web production by increasing consumption and respiration of fixed carbon within the microbial food web, and photosynthesis by protozoa increasing total production due to increased photosynthesis and

more efficient usage of ingested nutrients (C, N, P). When enhancing the total production within the microbial food web, mixotrophy should also enhance trophic transfer to metazoa, which should also depend on the relative quality/suitability of the mixotroph and its prey as food for metazoa (Stoecker 1998). Mixotrophy can also influence food web structure and function by affecting competition among phytoplankton (Rothhaupt 1996a, Stoecker et al. 1997, Thingstad et al. 1996) or by influencing predator-prey relationships within the microbial food web (Bockstahler & Coats 1993, Jeong et al. 1997, Uchida et al. 1997). Furthermore, the relative balance between different carbon and energy sources like DOC, inorganic nutrients, bacteria and light availability in the system is important for food web implications, where mixotrophs may become an important link in the flux of both carbon and phosphorus through the plankton community (Jones 2000).

Despite these important food web implications, research characterizing grazing and primary production in aquatic food webs was mainly focused on heterotrophic and phototrophic organisms, not taking mixotrophic feeding strategies into account (Sanders et al. 2000). The resulting lack of knowledge with regard to mixotrophy in many ecosystems is partly due to the fact that taxonomic composition of natural nanoflagellate assemblages is difficult to obtain. Furthermore, the taxonomic characterization does not necessarily provide information concerning phagotrophic activity of mixotrophic algae at any time or place because this activity can be induced or suppressed by a variety of conditions and/or limitations (see above). Identifying mixotrophic activity by algae in natural environments requires experimental evidence of feeding (Sanders et al. 2000). Fluorescently labeled bacteria (FLB) are commonly used as tracers to measure bacterivory in plankton communities (Sherr et al. 1987, Kemp 1988, Bloem et al. 1989, Epstein & Shiaris 1992, Hondeveld et al. 1992). FLB are added to the system and their uptake by predators can be quantified, either by enumeration of FLB in the food vacuoles of a predator or by the disappearance of FLB in the system due to grazing. The first method allows calculating grazing rates, clearance rates or ingestion rates whereas the latter permits the calculation of disappearance rates (e.g. Sherr & Sherr 1993, Borsheim 1984, McManus & Fuhrmann 1986, Pace & Baliff 1987). FLB grazing experiments are most commonly used to identify mixotrophs in natural environments and to estimate their grazing impact within the community (e.g. Berninger et al. 1992, Arenovski et al. 1995, Sanders et al. 2000, Havskum & Riemann 1996).

Given these methodological efforts and disparate potential benefits for species to be mixotroph, it has been difficult to formulate and test hypotheses with regard to mixotrophic distributions across a range of aquatic environments. There are, for instance, no records at all

about the occurrence or ecological impact of mixotrophs in benthic ecosystems. Compared to planktonic habitats, the ecological role of benthic protists is poorly investigated (Hondeveld et al. 1995, Wickham et al. 2000). Experimental investigations of benthic protists and their ecological function have been hampered particularly by methodological difficulties. Gradients of light, oxygen and redox potential are orders of magnitude steeper in sediment compared to the overlying water (Jørgensen & Revsbach 1985, Kühl et al. 1994, Berninger & Huettel 1997), requiring sampling on a very fine spatial scale. Beyond appropriate sampling techniques, further difficulties include the quantitative extraction of the organisms from their habitat and the inhomogeneity of marine and freshwater sediments (Bak & Nieuwland 1987, Bak & Nieuwland 1989, Hondeveld et al. 1994, Alongi 1993, Epstein 1995).

Hondeveld et al. (1995) and Starink et al. (1994, 1996a) have adapted the FLB method to measure the grazing pressure of benthic protists in sediments, however without taking mixotrophic protists into account.

In the present study this method is used and further modified to investigate the occurrence and the ecological impact of mixotrophic nanoflagellates in coastal marine sandy sediments. Due to the great variety of different functional types of mixotrophy in different organisms, whose adequate investigation would require numerous methods, many of which are still not optimal adapted for the use in sediments. Therefore, this study is confined to the investigation of mixotrophic nanoflagellates.

Shallow marine sediments harbor very diverse, abundant, and productive microbial assemblages (Fenchel 1969, Giere 1993). Micrograzers and microbial prey are present in abundances exceeding water column populations by one to several orders in magnitude. Mechanisms governing the dynamics of such assemblages may effectively control several processes of global importance. These processes include the remineralization of sedimented water column production, evolution and oxidation of reduced species such as $H_2S$, $NH_4^+$, $CH_4$, and the biogeochemistry of carbon, nitrogen, sulfur and other elements (Epstein 1997). Furthermore, shallow photosynthetically active sediments are unique in the sense that they can serve as a source of oxygen (Sundbäck et al. 1991, Lassen et al. 1992). Large populations of benthic phototrophic microorganisms, mostly diatoms, cyanobacteria and pigmented flagellates release oxygen into the overlying water and into the sediment (Revsbech et al. 1980, Revsbech & Jørgensen 1983, Yallop et al 1994, Reay et al. 1995). In contrast, the supply of oxygen originates solely from the water column in other seabeds, which are situated below the photic zone. The oxygen produced by benthic phototrophs is of pivotal importance for aerobic heterotrophic degradation processes within the sediment and for all aerobic

organisms associated with the sediment (Fenchel 1969, Cammen 1991, Sundbäck et al. 1991, Berninger & Epstein 1995).

Sediments are characterized by steep vertical and sometimes horizontal gradients of light, oxygen, nutrient concentrations and other physical and chemical factors (Jørgensen & Revsbech 1985, Kühl et al. 1994, Revsbech & Jørgensen 1986) that generate a pronounced heterogeneity. Due to the requirement of different physiological adaptations for the occupation of special ecological niches, gradients influence microbial community structure.

When regarding the ecological impact of mixotrophy in plankton communities, Jones (1994) suggested mixotrophy to be an adaptive strategy, which provides greater flexibility in the planktonic environment, which is prone to unpredictable spatial and temporal fluctuations. It was proposed that temporarily or spatially heterogeneous environments with respect to resources should particularly favor mixotrophs (Beaver & Crisman 1989, Berninger et al. 1986, Bird & Kalff 1987, Holen & Boraas 1995). Therefore, it can be assumed that similar food web implications can be found in heterogeneous environments like sediments.

In addition, a microbial sea ice community of an ice floe in the Greenland Sea was investigated. Sea ice is an important structural element of polar marine ecosystems (Horner et al. 1992, Thomas & Diekmann 2002). Frozen seawater consists of a semisolid matrix, which is permeated by a network of channels and pores, which vary in size from a few micrometers to millimeters. These channels and pores are filled with brine, which is formed from expelled salts as the ice crystals freeze together (Eicken 1992) and in which viruses, bacteria, algae, protists, flatworms and small crustaceans live. Sea ice is dominated by strong gradients of temperature, salinity, space and light (Thomas & Dieckmann 2002, Krembs et al. 2002). These properties as well as the morphology of the brine channel system are highly variable and are determined by air temperature and snow cover. These sea ice characteristics imply that the mixotrophic feeding strategy could also be an important survival strategy in this heterogeneous and variable system.

In the following chapters, a variety of studies are described that investigated different aspects of mixotrophic flagellates in a number of different systems with emphasis on coastal sediments. Different methods were used for FLB preparation in previous plankton and sediment studies. In Chapter 2, different FLB methods are described and tested to find the most efficient and realistic way for the quantitative identification of mixotrophic nanoflagellates (MNF) in coastal marine sandy sediments. Chapter 2 also provides the first records about the occurrence and the quantitative impact of MNF in coastal marine sediments. In the following (Chapter 3), mixotrophic feeding behavior in natural sediment communities

was investigated in dependence of environmental factors, which were shown to influence phagotrophic activity in mixotrophs (light and nutrient conditions, see above). This was done in order to investigate their ability to "switch" their nutritional modes in dependence of environmental factors and to estimate their potential role as bacterivores and primary producers within the benthic microbial food web under different environmental conditions in the laboratory.

Varying environmental conditions are found along vertical and also horizontal gradients in sediments, which can cause variations in microbial community structure along very small scales. Three different sediment types were investigated along a horizontal and a vertical gradient at Falckenstein Beach in the Western Baltic Sea to estimate the effect of small scale variations of physical and chemical parameters on microbial community dynamics with emphasis on mixotrophic nanoflagellates and their different feeding strategies. All experiments described in Chapters 2-4 were restricted to one particular location in Kiel Fjord in the Baltic Sea, where minor contributions of mixotrophic organisms were found. Due to physiological osmotic constraints of many organisms in the low saline Western Baltic Sea, it was assumed that energetical costs of mixotrophy (maintaining the photosynthetic apparatus as well as phagotrophy) could be too high for many species. In order to investigate whether mixotrophic feeding strategies play a greater role in fully marine or freshwater habitats, where organisms are not subject to osmotic stress, 5 different systems were investigated along a salinity gradient in Northern Germany in addition to 3 fully marine sites in the Pacific Ocean. Furthermore, microbial brine communities in sea ice of the Greenland Sea were investigated with respect to the quantitative importance of mixotrophs. This study allowed investigating whether previously found patterns in sediments of Falckenstein Beach can only be attributed to characteristics of that particular system in Kiel Fjord or have a general significance for coastal sediments. These system specific aspects are further discussed in Chapter 6, thereby outlining food web consequences of mixotrophy, its regulation and complex response as well as evolutionary aspects

# Chapter 2

## Comparison of different methods using fluorescent tracers to detect maximum abundances of mixotrophic nanoflagellates

### 2.1. Introduction

Microbial dynamics and the importance of microbial food webs has been subject of many plankton studies in recent years. In contrast, methodological difficulties have hampered the investigation of protists and their ecological function in benthic ecosystems (Hondeveld et al. 1995, Wickham et al. 2000). There is, for instance, no evidence for the occurrence or ecological impact of mixotrophic flagellates in benthic systems so far, although plankton studies have demonstrated their often pivotal role in microbial food webs (see Chapter 1).

The detection of mixotrophic flagellates requires the experimental evidence of feeding (Sanders et al. 2000). Fluorescently labeled bacteria (FLB) are commonly used to measure bacterivory in plankton communities (Sherr et al. 1987, Kemp 1988, Bloem et al. 1989, Epstein & Shiaris 1992, Hondeveld et al. 1992). One experimental design for these fluorescent tracer methods implies the determination of short term, cell-specific uptake rates via quantifying the average number of prey particles within protistan cells over a time course ranging from 10 to 60 min. (Sherr & Sherr 1993), after which prey (e.g. FLB) digestion begins (35 to 40 min. at 19°C, Sherr et al. 1988). An alternative approach using these prey particles is to follow the rate of disappearance of FLB over longer time periods (12h, 24h or more) (Sherr & Sherr 1993).

Hondeveld et al. (1995) and Starink et al. (1994a, 1996a) have adapted the FLB method to measure the grazing pressure of benthic protists in sediments. Hondeveld et al. (1992) estimated flagellate grazing directly in sediments, using natural bacterioplankton for FLB preparation. However, they noticed a high percentage of benthic protists without ingested FLB, which may result in underestimation of grazing rates. In contrast to those in the water column, bacteria in sediments are interstitial or attached to sediment particles (Starink et al. 1994a). Therefore, benthic flagellates are likely to have a preference for benthic bacteria, including attached and aggregate bacteria. Grazing preferences of surface-associated protozoa for attached bacteria have been demonstrated in batch cultures by Caron (1987) and Sibbald & Albright (1988). Starink et al. (1994a) developed a method using fluorescently stained

sediment versus monodispersed FLB, prepared out of a continuous culture inoculated with a mixed sample of sediment bacteria. They found twofold higher grazing rates with the fluorescently stained sediment in freshwater sediment from a littoral zone of Lake Gooimeer (Netherlands) compared to grazing rates determined with monodispersed FLB. According to Starink et al. (1994a), advantages of using fluorescently stained sediment include the following: 1) the ratio between attached and nonattached bacteria as well as the total amount of bacteria is not altered by mixing DTAF-stained sediment with fresh sediment and 2) the labeled bacterial community has the same structure as the non-labeled community, since all the sediment can be sampled from the same location. Many recent studies on heterotrophic grazing rates or the occurrence of mixotrophs, prepared FLB out of cultured bacterial strains and not from the target organism's natural prey (e.g. Arenovski et al. 1995, Sanders et al. 2000). In sediments, microbial communities are subject to seasonal changes and differ significantly at different sites, depending on sediment grain size, water depth, light and nutrient conditions etc. (Atlas & Bartha 1992, Findlay & Watling 1998). In the present study, I aimed at finding the most suitable and most realistic method for quantitative identification of mixotrophic nanoflagellates. Instead of using bacterioplankton for FLB preparation (Hondeveld et al. 1992) or bacterial cultures (Starink et al. 1994a) like in previous sediment studies, I modified the FLB method and isolated bacteria for FLB preparation out of sediment from the experimental sampling site directly before I conducted my experiments to account for the variation in bacterial prey. These natural monodispersed FLB were compared with fluorescently stained sediment (see Starink et al. 1994a) and FLB prepared from a bacterial culture of *Halomonas halodurans* (see Sanders et al. 2000). Furthermore, different incubation times with fluorescent tracers were tested. Experimental evidence showed that some mixotrophic species are able to respond to changing environmental conditions, i.e. to switch from photosynthesis to phagotrophy when light or nutrients are limiting (Nygaard & Tobiesen 1993, Keller et al. 1994, Urabe et al. 1999, Urabe et al. 2000). Since my experiments were designed to assess maximum abundances of potentially mixotrophic flagellates, part of the sediment in Experiments I and II and all samples in Experiment III and IV were incubated in the dark. This was done to induce a switch from photosynthesis to phagotrophy in some mixotrophic phytoflagellates, promoting FLB grazing and allowing me to identify more phytoflagellates as mixotrophs than in light incubations. Beyond the comparison of established methods using different fluorescent tracers, this study provides a further modification of the FLB method for the use in sandy sediments, and presents first data on the

quantitative and qualitative importance of mixotrophic nanoflagellates in coastal marine sediments.



## 2.2. Material and Methods

### 2.2.1. Experiments and sampling site

Four grazing experiments were conducted. Experiment I-III were conducted with sediment from Falckenstein Beach, Kiel Fjord, Western Baltic Sea (10° 11' 40''E, 54° 24' 23''N) in September 2000, October 2000 and January 2001, respectively. The experiment conducted in the beginning of September presents late summer conditions within the vegetation period, whereas experiments conducted in late October and in January present autumn and winter conditions. Due to low light conditions and stormy weather, causing water movement and disturbing sediment surface layers, conditions were assumed to be unfavorable for phytoflagellates. Therefore, mixotrophy was expected to be an important feeding strategy at this time of the year.

Kiel Fjord is an extension of Kiel Bight, a shallow water area with an average water depth of 18-20m. It is located between the fully marine Kattegat (30 - 35psu) and the low saline Central Baltic Sea (7 psu). The salinity in Kiel Fjord ranges from 15 to 21 psu throughout the year. The climate of the Western Baltic is temperate and humid with annual water temperatures in surface waters ranging from 22° in July/August to 0.5-1°C in December/January. The Western Baltic Sea exhibits, due to its small size, almost no tides and the tide-related diurnal water-level amplitude lies below 15cm (Lass & Magaard 1995). However, this amplitude can be exceeded by wind-driven changes in water-level. The sediment I investigated at Falckenstein Beach was coarse sediment, with more than 60% of its grains being > 1mm (see Fig. 4.1., station 1 in Chapter 4). Organic matter contributed approximately 1% to the total sediment (see Fig. 4.2., station 1 in Chapter 4). Sediment cores were taken at a water depth of 0.3 - 0.5m, 2m below the shore line. In order to work in the euphotic zone with aerobic organisms, only the sediment surface layer of 3mm from each core was used for the experiments.

Experiment IV was conducted with shallow plankton from Newport Beach (33°36'16''N, 117°55'28''W) and Huntington Beach (33°39'46''N, 118°0'43''W) two fully marine locations (29-34psu) south of Los Angeles in Southern California in March 2003.

*Fluorescently labeled sediment*

The fluorescently labeled sediment was prepared in correspondence to Starink et al. (1994a) in the week before the grazing experiment. In the following text it is called fluorescently labeled sediment, FLS, and the FLB out of the FLS are called $FLB_{FLS}$. Sediment was collected at the sampling site at Falckenstein Beach and processed in the laboratory. It was stained with DTAF at a final concentration of 0.2g $l^{-1}$ and then incubated in a water bath for 4h at 70°C, with a shallow supernatant of seawater to keep it wet. After DTAF addition and every 30 min., the stained sediment was stirred with a spoon to allow for even staining. After incubation, the FLS was washed several times to dispose the remaining DTAF, i.e. 10ml SSW were added, the suspension vortexed and then centrifuged for 20 min. at 13500rpm and 5°C, until the supernatant was clear. The sediment was collected in polypropylene centrifuge tubes and frozen at -20°C. After determining $FLB_{FLS}$ concentrations in the FLS, the required amount of sediment was directly thawed before use in a grazing experiment.

*Fluorescently labeled culture bacteria*

Bacteria from the culture *Halomonas halodurans* (supplied courtesy of D. Caron, University of Southern California, Los Angeles, USA) were directly concentrated via centrifugation and then processed in the same way as the natural bacterial suspension. FLB concentrations ($FLB_{Hal}$) were determined in the stock solution and FLB was frozen at -20°C until used in the experiment.

### 2.2.3. Sampling procedure

Sediment was collected with acrylic glass cores, 2mm thick and 20cm in length with an inner diameter of 2.5 cm. They were taken at a water depth of 0.1 – 0.3m. Before sampling, the beach was divided into 20 patches. Four patches were randomly selected to take four replicate cores for every treatment and census, respectively (Table 2.1.), to account for the heterogeneity of the sediment along the shore. Cores were collected in a cooler with frigistors and plankton in 20l polyethylene containers. Samples were taken immediately to the laboratory for further processing. Sampling procedure and processing of a FLB grazing experiment with sediment is illustrated in Fig. 2.1.

*Sediment:*

The first 3mm of surface sediment were extruded from each core, which was equivalent to 1.5$cm^3$ +/- 0.4 $cm^3$ of sediment. Sediment slices were transferred into wells of a tissue culture plate. The sediment density was determined by adding 1g of sediment to a measuring cylinder

filled with 10ml of water. The water displacement was measured and the density calculated. Each sediment slice was weighed to calculate accurate sediment volumes for each sample afterwards.

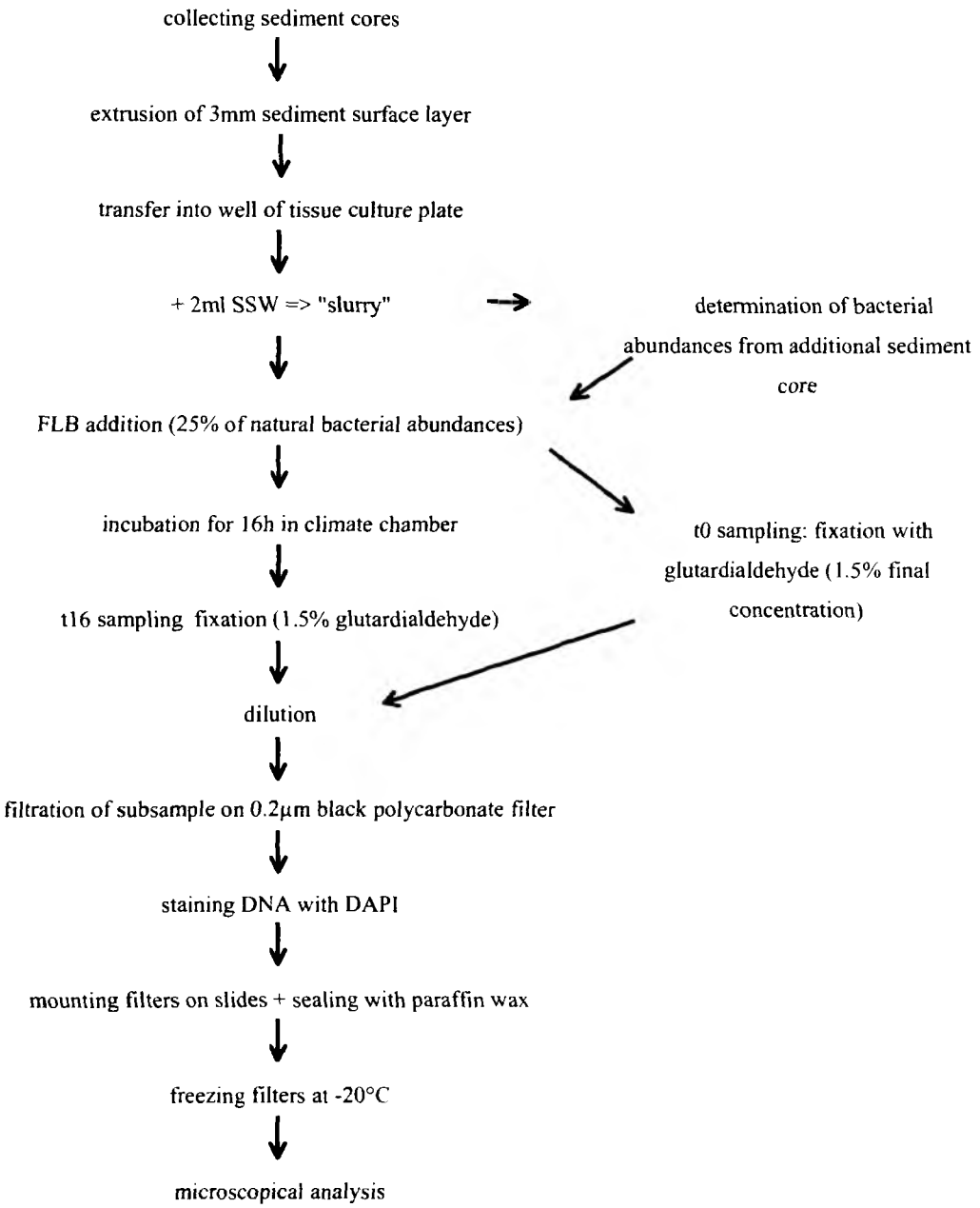



**Fig. 2.1.** Working process of a FLB grazing experiment in sediment, considering an incubation time of 16h as employed in Experiments I ($FLB_{nat\,sed}$), II ($FLB_{nat\,sed}$) and IV ($FLB_{lfal}$).

Each well contained 2ml of SSW and the sediment cut was transferred into slurry. From one additional sediment core, bacterial abundances were determined (see below) to calculate

FLB concentrations to be added to the slurries (equal to 25% of natural bacterial abundances, see Sherr & Sherr. 1993). The FLB stock solution was thawed and diluted with SSW, so that 1ml of working solution was equal to the required amount of FLB. The working solution was sonicated (3 x 30 sec. at 30W power-level), so that FLB would not clump and evenly disperse in the sample. After adding FLB, samples were gently shaken and then t0 samples were fixed with ice-cold glutardialdehyde at a final concentration of 1%, in order to prevent egestion of food vacuole content upon fixation (Bloem et al. 1988). Samples were stored at 4°C until further processing. T0 samples presented the first set of replicates, 4 for each treatment that had to be initially established.

Incubations with fluorescently labeled sediment (FLS) were processed in a similar way. After determining bacterial abundances in the natural sediment, the amount of FLS, containing FLB abundances equal to 25% of the natural bacterial abundances, was calculated. FLS was thawed, gently stirred with a spoon and then added with a spatula to the samples, which were again gently, but thoroughly stirred.

After fixation of t0 samples, dark incubations were completely covered with aluminum foil and then all samples were incubated in a temperature controlled room (18°C) with a light/dark-cycle of 16:8 hours and a light intensity of 30 - 35 $\mu E\ m^{-2}\ s^{-1}$ (measured with LI-COR LI-189). After 16 hours of incubation (Table 2.1.) the samples of Experiment I and II (the remaining set of replicates) were fixed with ice-cold glutardialdehyde (1.5%). In Experiment III, one set of replicates was fixed after, 6h, 16h, 24h and 48h incubation time, respectively (Table 2.1.).

*Plankton*

Water samples were collected in 20l polyethylene containers at two locations south of Los Angeles in Huntington Beach (HB) and Newport Beach (NB). In the laboratory, plankton was filtered through 200µm gauze to exclude meso- and macrozooplankton species. They would have influenced my incubation bottles differently by uneven grazing due to uneven species distribution in the 1l sampling bottles. Samples were immediately filled into 1l polycarbonate bottles. At first, the bottles were not completely filled, because FLB still had to be added and mixed within the sample. After determining natural bacterial abundances in the plankton from both locations (see below) the amounts of FLB to be added were calculated (25% of the natural bacterial abundances). FLB stock solutions were diluted so that 5ml of working solution was equal to the FLB concentration to be added to the samples. This was done to assure better mixing of FLB within the plankton sample. After sonicating the FLB working solutions, FLB were added to the samples and thoroughly shaken. The bottles were

completely filled up with plankton. Again, treatments were replicated 4 times (Table 2.1.). However, sampling was not destructive and t0 and t16 samples could be taken out of the same bottles, resulting in a total of 4 bottles per sampling site. For t0 sampling, a subsample of 27ml was fixed for all replicates of each treatment with 3ml of 10% ice cold Glutardialdehyde and stored at 4°C. Afterwards, the bottles were filled up with SSW and then incubated in dark boxes in a climate chamber under the same conditions as described above. After 16h, t16 samples were taken in the same way as t0 samples.

### 2.2.4. Sample processing

*Sediment*

Fixed sediment samples were "washed out" of the cell-wells by adding 5ml of SSW at a time with a 5ml pipette (uncut pipette tip, exact volume) and transferring the SSW-sediment suspension into a 300ml polystyrene cell culture flask by using a 5 ml pipette with a cut pipette tip. After the sediment sample was completely "washed out" of the cell-well, i.e. SSW added and transferring SSW-sediment suspensions into the culture flask, until no sediment was left in the well, the SSW-sediment suspension was diluted with SSW to a total volume of 150ml and the suspension thoroughly shaken. After the biggest sand grains had settled down, 20ml of the supernatant was fixed with glutardialdehyde at a final concentration of 1.5%. Since the sediment was very sandy and did not consist of very fine flocculent material, it was not necessary to extract the organisms via density gradient centrifugation. This method of extracting the organisms out of the sediment only by dilution was tested in preliminary experiments and was more efficient than sonicating the sample. Sonicating fragmented sediment particles and organisms more than it separated them, and flagellates could only be detected at very high dilution of the samples.

Depending on species abundances and organic material content, a certain volume of this subsample was collected on a 0.2µm polycarbonate filter (Nucleopore Track-Etch Membrane, PC MB 25mm 0.2µm) and stained with DAPI (4', 6-Diamidino-2-phenylindol) for 5min at a final concentration of 5 µg/ml, to stain DNA of bacteria and protists (Porter & Feig 1980, Sherr & Sherr 1993). Filters were sealed between a slide and cover-slip with paraffin wax and stored at -20°C until analysis. For an overview of the working processes of a FLB grazing experiment, see Fig. 2.1.

*Plankton*

Fixed plankton samples could directly be filtered on polycarbonate membranes. Subsamples of 1ml were filtered on 0.2µm polycarbonate filters for counting bacteria, flagellates, diatoms, cyanobacteria and FLB. Again, samples were stained with DAPI for 5min, mounted on slides, sealed with paraffin wax and stored at -20°C until analysis.

### 2.2.5. Counting

Filters were counted with an epifluorescence microscope (Leica/Leitz DMRB) at 1000x magnification. Flagellates were counted under blue light in 60-80 fields of view. Flagellates and diatoms were counted using a blue filter set (450-490nm excitation, >515nm emission), where phototrophic and heterotrophic nanoflagellates (PNF and HNF) were differentiated by the presence/absence of chlorophyll autofluorescence using a blue filter set (450-490nm excitation, >515nm emission, Leica/Leitz filter set 13). DAPI fluorescing protistan nuclei and bacteria were visible and counted using a UV filter set (340-380nm excitation, >420nm emission, Leica/Leitz filter set A). Due to DAPI staining of nuclei, flagellates could be distinguished from debris and other particles. Mixotrophic nanoflagellates (MNF) were defined as cells containing autofluorescent chloroplasts and 1 or more ingested FLB, which were visible in the flagellates as green fluorescing rods and cocci. However, it was not possible to count FLB within the species themselves, since FLB were sometimes only visible as big green fluorescing clumps within the flagellates. Therefore, it was not possible to calculate grazing rates from direct FLB ingestion. Furthermore, diatoms, FLB and bacteria were counted; diatoms in 60-80 fields of view, FLB and bacteria in 12 fields of view.

### 2.2.6. Statistical Analysis

In order to test for differences between light and dark incubations in Experiments I and II, a one-factor ANOVA on abundances of mixotrophs was conducted. Normal distribution and homogeneity of variances were tested with a Chi-Square-Test and with a Bartlett-Chi-Square-Test, respectively. Posthoc tests were conducted with Tukey's HSD test. To analyze differences in incubation time and the use of $FLB_{nat/sed.}$ versus $FLB_{FLS}$ in Experiment III, a two-factor ANOVA on abundances of mixotrophs was conducted (incubation time x fluorescent tracer). To analyze differences in FLB grazing ($FLB_{nat/sed.}$ versus $FLB_{FLS}$) over time, a linear multiple regression analysis was applied on FLB abundances (incubation time x fluorescent tracer). A two-factor ANOVA on abundances of mixotrophs was conducted to

analyse differences in location (Huntington Beach/Newport Beach) and FLB ($FLB_{nat/plank.}/FLB_{Hal.}$).

## 2.3. Results

The microbial community in the sediment of Falckenstein Beach differed noticeably between September (Experiments I ($FLB_{nat/sed}$)) and October 2000 (Experiment II ($FLB_{nat/sed}$) (Fig. 2.2.). In October 2000, abundances of all nanoflagellates and diatoms were approximately one decimal power higher than in September (Fig. 2.2.). Only bacterial abundances were in the same range of $1x10^{10}/cm^3$. In Experiment III (FLS, January 2001), microbial abundances approximated abundances in September 2000 (Experiment I ($FLB_{nat/sed}$)), except for bacteria, which only reached abundances of $1x10^8/cm^3$ (Fig. 2.2.). In plankton communities in Experiment IV ($FLB_{Hal.}$), abundances of HNF, PNF, MNF and diatoms ranged from $1x10^2$ to $1x10^4/cm^3$, bacterial abundances from $1x10^6$ to $1x10^7/cm^3$.

In all experiments, the nanoflagellate community was clearly dominated by heterotrophic nanoflagellates (HNF) (Fig. 2.3.), contributing 65-80% to the total nanoflagellates. With increasing percentage of phototrophic nanoflagellates (PNF) from Experiment I to IV, also the percentages of mixotrophic nanoflagellates (MNF) increased from 1.5% to 4.4% (Fig. 2.3., Table 2.2.). Grazing activity of HNF, i.e. HNF with ingested FLB as percentage of the total HNF, ranged from 8% to 20% (Table 2.2.).

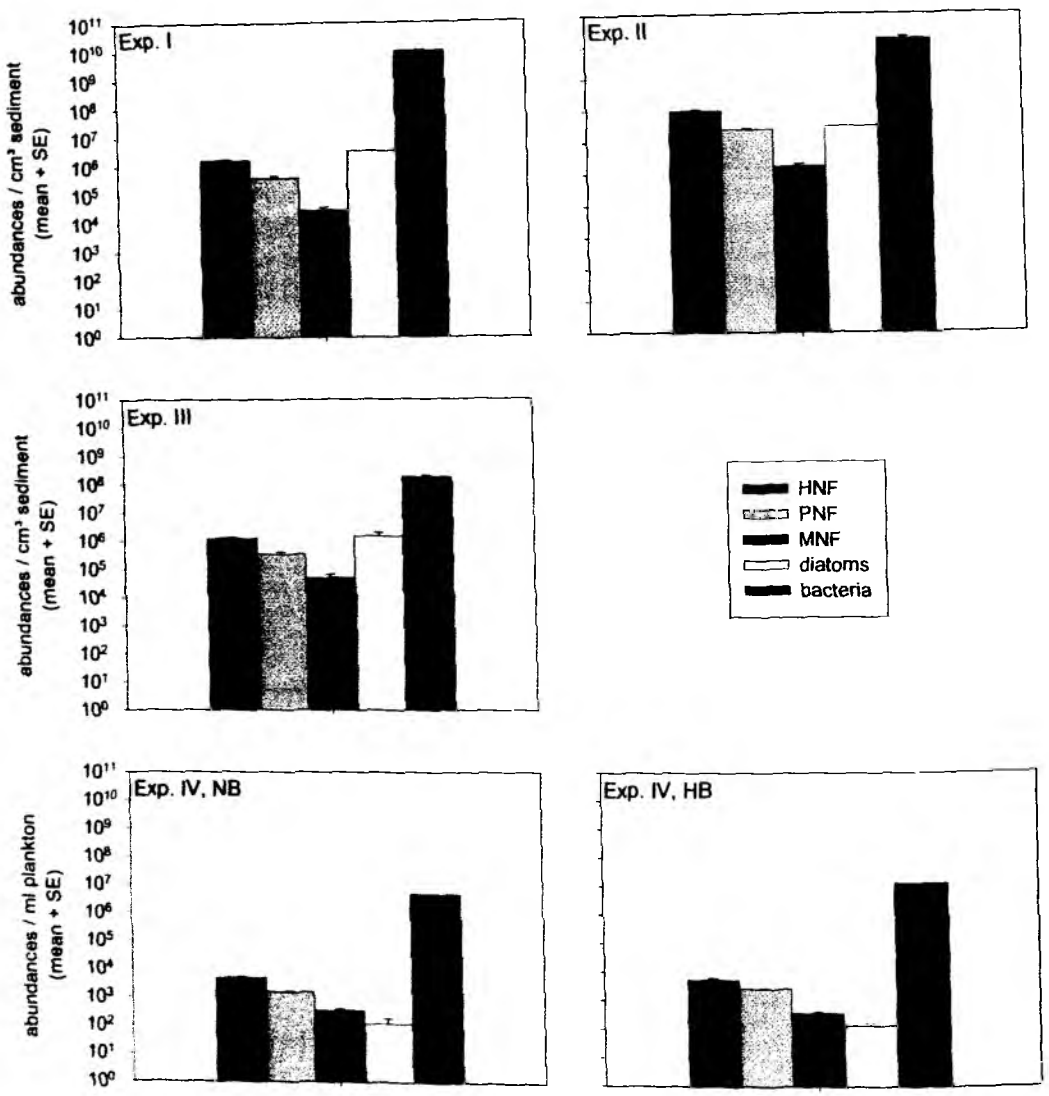



**Fig. 2.2.** Microbial community structure in different sediments of Falckenstein Beach (Experiment I, II and III) and plankton of Newport Beach (NB) and Huntington Beach (HB), (Experiment IV). Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophic nanoflagellates (MNF), diatoms and bacteria per $cm^3$ sediment in dark incubations with natural $FLB_{nat}$. In Experiment IV, abundances after 16h of incubation time are presented.

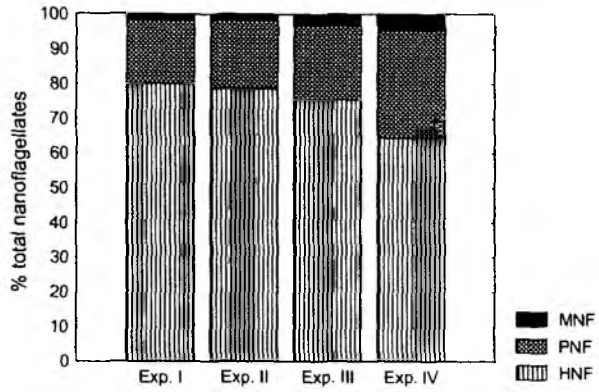



**Fig. 2.3.** Relative abundances of heterotrophic (HNF), phototrophic (PNF) and mixotrophic (MNF) nanoflagellates as percentage of the total nanoflagellates in Experiments I to IV incubated with natural FLB. In Experiment I and II, communities in dark incubations are presented. In Experiment IV, abundances after 16h of incubation time are presented.

| Experiment | % MNF / NF | % HNF+FLB / total HNF |
|---|---|---|
| **I dark** | 1,43 ± 0.4 | 19.2 ± 2.4 |
| **I light** | 0.21 ± 0.18 | 9.3 ± 2.1 |
| **II dark** | 1.4 ± 0.3 | 11.8 ± 1.0 |
| **II light** | 0.69 ± 0.1 | 10.7 ± 0.8 |
| **III +FLB** | 2.2 ± 0.5 | 7.7 ± 0.6 |
| **III +FLS** | 1.4 ± 0.3 | 5.0 ± 1.1 |
| **IV Newport Beach** | 5.2 ± 0.3 | 12.5 ± 0.9 |
| **Huntington Beach** | 4.5 ± 0.4 | 10.4 ± 1.1 |

**Table 2.2.** Relative abundances of mixotrophic nanoflagellates as % of the total nanoflagellates (%MNF / NF) and heterotrophic nanoflagellates with ingested FLB as % of the total heterotrophic nanoflagellates (%HNF + FLB / total NF) for all Experiments I – IV. Values in Experiment I and II refer to dark incubations. Values in Experiment III are averaged over all incubation times. Values in Experiment IV refer to the use of natural FLB. Values present mean ($n$=4) ± standard error.

### 2.3.1. Experiment I and II ($FLB_{nat/sed}$):

In October (Experiment II), absolute abundances of MNF exceeded those in September (Experiment I) by the factor 5 (Fig. 2.4.). However, relative abundances of MNF as percentage of the total nanoflagellates were almost equal in both experiments and ranged from 0.5% in the light to 1.4% in the dark (Table 2.2.). Mixotrophs contributed a maximum share of 2% to the total bacterivorous grazers (HNF+MNF), and up to 8% to the total phytoflagellates (Fig. 2.5.). In both experiments, significantly more mixotrophs with ingested FLB were found in dark incubations compared to the light (Fig. 2.4., Table 2.3.A), indicating that part of the mixotrophic community was able to switch from photosynthesis to

phagotrophy under light limitation. In October, this effect was significant at a level of p=0.051. In Experiment I, HNF showed higher grazing activities in dark incubations compared to the light, with almost 20% HNF with ingested FLB in the dark compared to only 10% in light incubations. This effect was not evident in Experiment II, where in both light and dark incubations 11-12% of the total HNF ingested FLB (Table 2.2.).

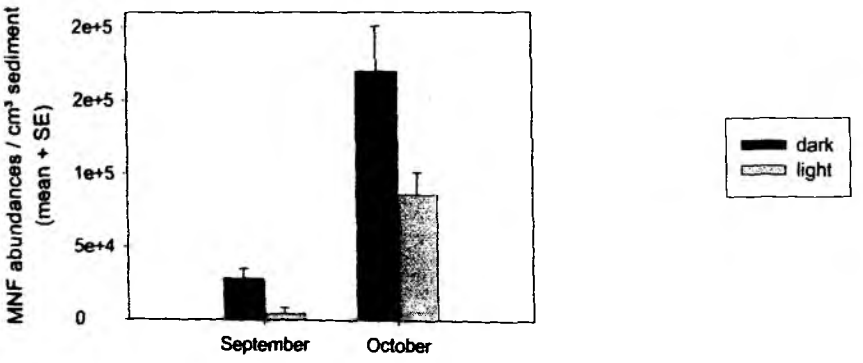



**Fig. 2.4.** Absolute abundances of mixotrophic nanoflagellates (MNF) per cm³ sediment in September (Exp. I) and October (Exp. II) 2000 at Falckenstein Beach.

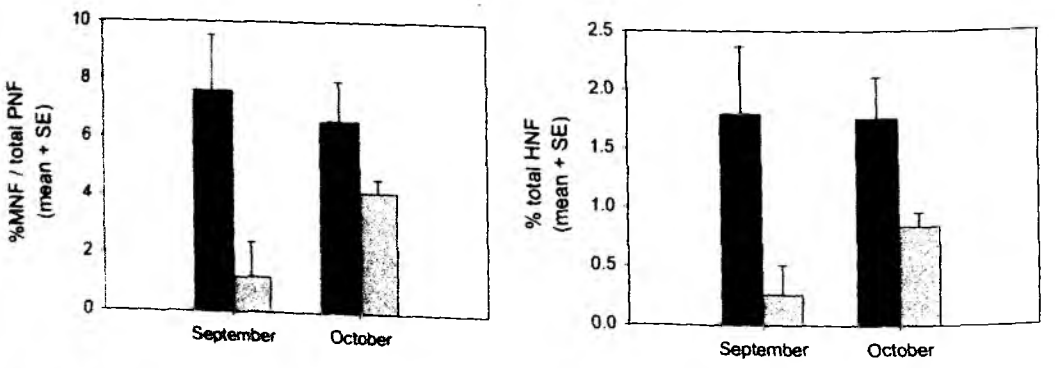



**Fig. 2.5.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates (PNF) and the total heterotrophic nanoflagellates (HNF) in September and October 2000 (Experiment I and II). Note the different scalings on the y-axes.

| Factor | Experiment I | Experiment II |
|---|---|---|
| **df** | 6 | 6 |
| **light/dark (1)** | 9.335<br>**(0.022)** | 5.948<br>*(0.051)* |

(B)

| Factor | mixotrophs | heterotrophs |
|---|---|---|
| **df** | 30 | 30 |
| **time (4)** | 4.419<br>**(0.006)** | 0.778<br>(0.548) |
| **fluorescent tracer (1)** | 0.114<br>(0.738) | 3.259<br>*(0.081)* |
| **time x tracer (4)** | 3.639<br>**(0.016)** | 0.787<br>(0.543) |

(C)

| Factor | mixotrophs | heterotrophs |
|---|---|---|
| **df** | 12 | 12 |
| **location (1)** | 1.537<br>(0.239) | 5.559<br>**(0.036)** |
| **fluorescent tracer (1)** | 0.730<br>(0.410) | 2.100<br>(0.173) |
| **location x tracer (1)** | 0.184<br>(0.676) | 7.445<br>**(0.018)** |

**Table 2.3.** Results of a one factor **ANOVA** on abundances of mixotrophs (MNF) and heterotrophs (HNF) with ingested FLB. The table gives the F-ratios (with significance levels in parentheses) for the main factors for all experiments. The degrees of freedom for the effect terms are given in parentheses for each effect, for the error term in the row on top of the analysis. Effects significant at $p<0.05$ are printed in bold, trends with $p<0.1$ are printed in italics. (A) One-factor ANOVA on abundances of mixotrophs in Experiments I and II (light/dark). (B) Two-factor ANOVA (incubation time x fluorescent tracer (FLB/FLS)) on abundances of MNF and HNF with ingested FLB. Results should be considered with care since a violation of variance homogeneity was detected (MNF: Bartlett's $\chi^2=17.524$, $p=0.042$; HNF: Bartlett's $\chi^2=20.594$, $p=0.015$) and data were not normally distributed for HNF ($\chi^2=15.194$, $p=0.019$). (C) Two-factor ANOVA (location (Newport Beach / Huntington Beach) x fluorescent tracer ($FLB_{Nal}$/ $FLB_{Hal}$)) on abundances of MNF and HNF with ingested FLB. Results should be considered with care since data were not normally distributed for MNF ($\chi^2=7.175$, $p=0.028$).

### 2.3.2. Experiment III (FLS)

In January 2001, $5x10^4$ to more than $10^5$ MNF/cm³ sediment with ingested FLB were detected at Falckenstein Beach (Fig. 2.6.), minimum numbers in the same range as in Sep. 2001 (Exp. I) and maximum numbers as in Oct. 2001 (Exp. II). They only contributed about 1% to the total nanoflagellates (Table 2.2.), but up to 5% to the total HNF and even up to 15% to the total PNF (Fig. 2.7.). Abundances of MNF with ingested FLB varied significantly with incubation time (Table 2.3.B, Fig. 2.6.), whereas they did not vary significantly with the use

of different fluorescent tracers ($FLB_{nat/sed.}$ and $FLB_{FLS}$). The interaction between the two factors was significant (Table 2.3.B), indicating that incubation time had disparate effects on the ingestion of different tracers (Table 2.3.B). The abundances of mixotrophs with ingested $FLB_{FLS}$ after 0.5h were significantly lower than with ingested $FLB_{nat/sed.}$ after 0.5h, and also lower as MNF abundances with both ingested $FLB_{FLS}$ and $FLB_{nat/sed.}$ after 16h (Fig. 2.5.), (Tukey's HSD, p(t0 FLB) = 0.05), p(t16 FLS) = 0.001, p(t16 FLB) = 0.024). In contrast to FLB ingestion by mixotrophs, neither incubation time nor the use of different fluorescent tracers or the interaction between both factors had any effect on the FLB ingestion of HNF (Figure 2.6., Table 2.3.B).

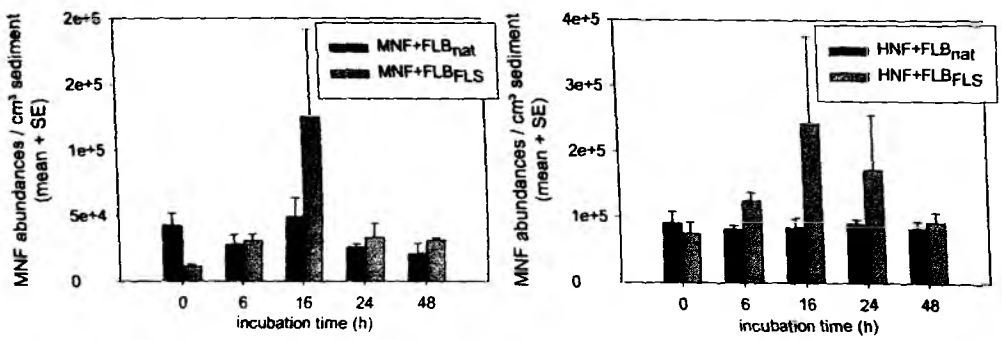



**Fig. 2.6.** Absolute abundances of mixotrophic nanoflagellates (MNF) and heterotrophic nanoflagellates (HNF) per cm³ sediment with ingested natural monodispersed FLB ($FLB_{nat}$) and FLB out of the fluorescently labeled sediment ($FLB_{FLS}$) respectively. Note the different scalings on the y-axes.

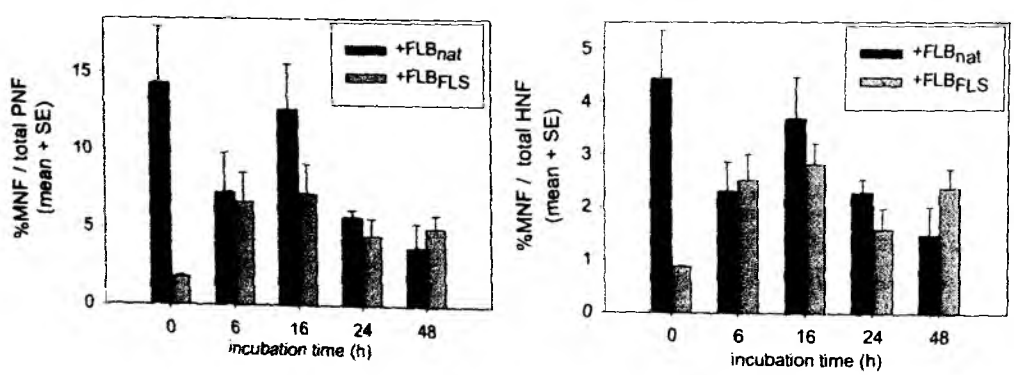



**Fig. 2.7.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates (PNF) and the total heterotrophic nanoflagellates (HNF) in Experiment III. Note the different scalings on the y-axes.

Grazing activity of HNF was lower than in Experiment I and II; only 8% of the total HNF were found with ingested FLB.

In consequence of grazing, $FLB_{nat/sed.}$ and $FLB_{FLS}$ abundances significantly decreased with increasing incubation time (Table 2.4., Fig. 2.8.). The regression line of $FLB_{nat/sed.}$ was significantly steeper than the $FLB_{FLS}$ regression line (Table 2.4.), indicating a stronger grazing on $FLB_{nat/sed.}$. $FLB_{FLS}$ decreased to approximately 70% of initially added abundances, whereas $FLB_{nat/sed.}$ was grazed down to appr. 40% (Fig. 2.8.).

| N=40 | B | St. Error of B | p-level |
|---|---|---|---|
| **Intercept** | $-1.033 \times 10^{10}$ | $1.277 \times 10^{9}$ | <0.001 |
| **incubation time** | $-9.778 \times 10^{5}$ | $3.791 \times 10^{5}$ | 0.014 |
| **fluorescent tracer** | $1.041 \times 10^{8}$ | $1.271 \times 10^{7}$ | <0.001 |

**Table 2.4.** Results of a linear multiple regression analysis of the effect of incubation time and fluorescent tracer ($FLB_{mono}/FLB_{FLS}$) on FLB abundances. Adjusted $R^2 = 0.648$, $F(2, 37) = 36.846$, $p<0.001$.

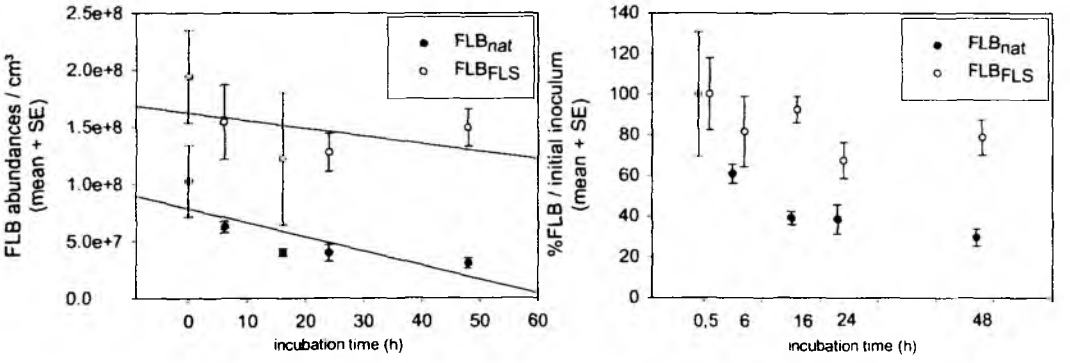



**Fig. 2.8.** Concentrations of natural FLB ($FLB_{nat}$) and FLB out of fluorescently labeled sediment ($FLB_{FLS}$) in the time course of Experiment III as absolute abundances and as percentage of the initial FLB inoculum (FLB t0 = 100%).

### 2.3.3. Experiment IV

In plankton communities of Newport Beach and Huntington Beach, 400-600 MNF with ingested FLB were detected per ml plankton (Fig. 2.9.). They contributed 5% to the total nanoflagellates (Table 2.2.), accounting for up to 7% of the total bacterivorous grazers (HNF and MNF+FLB) and for up to 18% of the total phytoflagellates (Fig. 2.10.). Abundances of MNF with ingested FLB did neither differ significantly with location (Newport Beach, Huntington Beach) nor with the use of different fluorescent tracers (Table 2.3.C, Fig. 2.9.).

Grazing activities of HNF (abundances of HNF with ingested FLB) differed significantly with location, but not with the use of different tracers (Table 2.3.C). The interaction between the factors was significant, too, (Table 2.3.C), with abundances of HNF+$FLB_{Hal}$ being significantly lower in Newport Beach than in Huntington Beach (Fig. 2.8., Tukey's HSD, p=0.017).

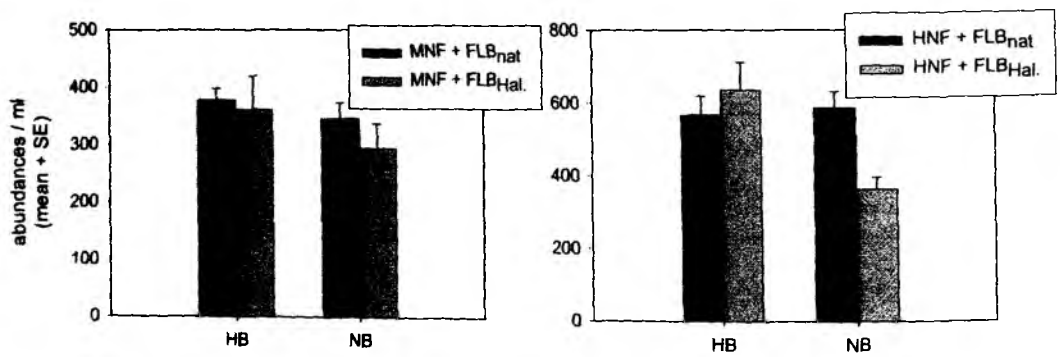



**Fig. 2.9.** Absolute abundances of mixotrophic nanoflagellates (MNF) and heterotrophic nanoflagellates (HNF) per ml plankton with ingested $FLB_{nat}$ and $FLB_{Hal.}$, respectively, in Experiment IV in Huntington Beach (HB) and Newport Beach (NB). Note the different scalings on the y-axes.

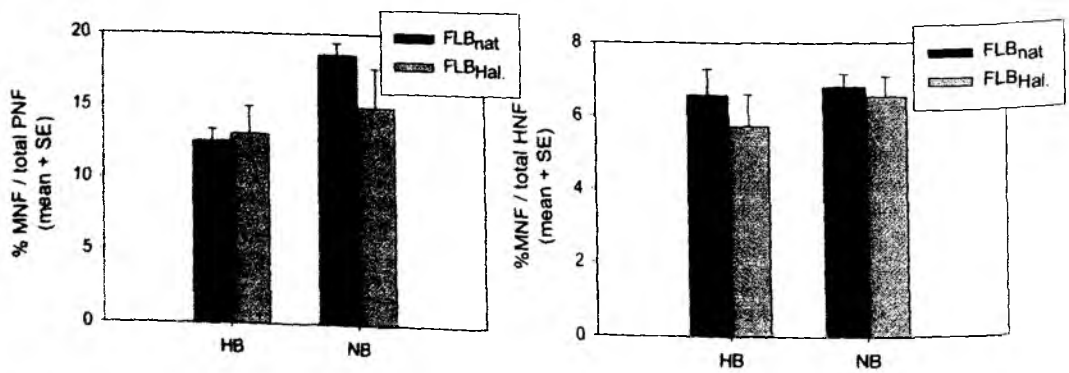



**Fig. 2.10.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates (PNF) and the total heterotrophic nanoflagellates (HNF) in Experiment IV. Note the different scalings on the y-axes.

Overall, grazing on FLB was clearly stronger in Huntington Beach than in Newport Beach (Fig. 2.11.); in Huntington Beach, both $FLB_{nat/plank.}$ and $FLB_{Hal.}$ were grazed down to 20% of initially added FLB. In Newport Beach, $FLB_{nat/plank.}$ were only grazed down to approximately 70%, $FLB_{Hal.}$ down to 40% (Fig. 2.11.).

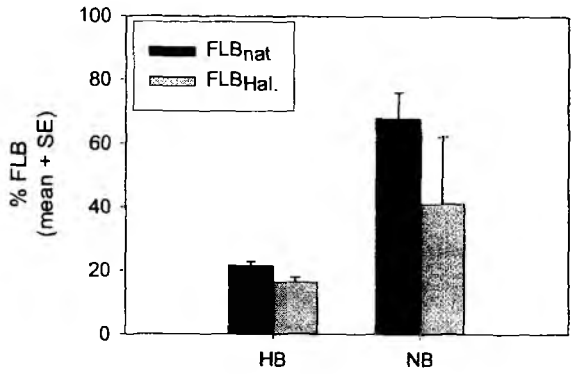



**Fig. 2.11.** $FLB_{nat}$ and $FLB_{Hal}$ concentrations in Experiment IV in Huntington Beach (HB) and Newport Beach (NB) after an incubation time of 16h as percentage of the initial FLB inoculum (FLB t0 = 100%).

## 2.4. Discussion

In my experiments, I was able to detect mixotrophic nanoflagellates (MNF) in coastal marine sediments of Falckenstein Beach and also in plankton communities of coastal shallow waters at Newport Beach and Huntington Beach in Southern California.

For FLB grazing experiments in sediments it can be assumed that slurrying of the shallow superficial layers of sediment, which are normally subject to resuspension due to water movement, introduced no measurable artifacts in measurements of flagellate ingestion rates (Kemp 1988). My study presents the first record of benthic mixotrophic nanoflagellates in natural sediment communities. MNF contributed a maximum share of 2% to the total nanoflagellates in sediments. All communities were clearly dominated by HNF and MNF only contributed up to 5% to the total bacterivorous grazers (HNF+MNF) and up to 17% to the total phytoflagellates. They played a considerable role as primary producers, but a minor role as bacterivores, not controlling bacterial abundances. The shallow plankton communities investigated in this study, were also dominated by HNF, although not to the same extent as the sediments. Here, MNF contributed 5% to the total nanoflagellates, 7% to the total heterotrophs (HNF+MNF) and almost 20% to the total phytoflagellates, thus playing a similar role as in investigated sediment communities.

Heterotrophic grazing activity varied considerably in Falckenstein sediments at different time points, being lowest in January, whereas phagotrophic activity in mixotrophs hardly changed with time. Apart from seasonal patterns (e.g. Starink et al. 1994a, Hondeveld et al. 1994), HNF grazing activity is dependent on protozoan cell size and abundance, bacterial

abundance and production and on temperature (e.g. 1988, McManus & Fuhrmann 1988, Bennett et al. 1990, Vaqué et al. 1994). In contrast, a variety of other environmental factors such as light level, nutrient concentrations etc. determine mixotrophic feeding behavior (Nygaard & Tobiesen 1993, Keller et al. 1994, Urabe et al. 1999, Urabe et al. 2000).

I was not able to count ingested FLB in the flagellates themselves; therefore I could not directly calculate grazing rates for MNF and HNF. In natural sediment communities it is also not possible to calculate grazing rates from FLB disappearance, since nanoflagellates do not present the major bacterivores in this system with the highest grazing impact on bacteria. Epstein (1997a) investigated FLB ingestion rates in different groups of bacterivorous grazers. The contributions if individual groups were similar, with 30% nanobenthos, 42% microbenthos (21% microflagellates, 21% ciliates) and 28% meiobenthos. However, the ingestion rates were lowest in pigmented nano- and microflagellates and were highest in pleurostomatid ciliates and nematodes. Ciliates and nematodes were not investigated in this study and their grazing impact on bacteria can not be estimated in the systems I investigated.

Beyond providing first data on the quantitative and qualitative importance of mixotrophic nanoflagellates in coastal marine sediments, my experiments aimed at finding the most efficient method to identify maximum abundances of MNF by using fluorescent tracers. Every type of FLB used in my experiments was ingested by both HNF and MNF. The modified FLB method using monodispersed natural FLB with an incubation time of 16h, proved to be the most efficient for the systems I investigated; this kind of FLB was decimated most by grazing and most MNF with ingested FLB were found in these incubations. More mixotrophs with ingested FLB were found in dark incubations compared to the light/dark cycle, indicating that part of the mixotrophic community was able to switch from photosynthesis to phagotrophy under light limitation. This treatment was retained in subsequent experiments to detect higher abundances of potentially mixotrophic species (Chapter 3-5).

Starink et al. (1994a) found twofold higher grazing rates in heterotrophs using fluorescently stained sediment compared to monodispersed FLB. They assumed that phagotrophic benthic protists have a wide range of feeding strategies in order to maximize niche segregation, some species having a marked ability to browse particle attached bacteria and others being more adapted to browse suspended bacteria. However, they used bacterial cultures for preparation of monodispersed FLB instead of natural bacteria out of the sediment. In my experiments, there were no significant differences between the abundances of MNF with either $FLB_{FLS}$ or $FLB_{nat'sed.}$. Monodispersed natural FLB were grazed to a greater extent

than the mixture of attached and interstitial FLB out of the FLS. The sediment I investigated was very sandy (Fig. 4.1., station 1 in Chapter 4). The sediment cores were taken only 2m behind the shore line in very shallow water of 0.3 to 0.5m depth. In this zone, the sediment surface layer is continuously resuspended due to water movement. In this environment, flagellates might be more adapted to graze on free bacteria than on attached bacteria compared to the fine freshwater sediment Starink et al. (1994a) collected from a littoral zone of Lake Gooimer. In my experiment it was shown that after 0.5h there were significantly more MNF with ingested $FLB_{nat/sed.}$ compared to $FLB_{FLS}$, indicating that MNF preferred freely accessible monodispersed FLB at first. After that until the end of the experiment after 48h, equal portions of MNF were found with $FLB_{nat/sed.}$ and $FLB_{FLS}$. However, significantly more $FLB_{nat/sed.}$ were grazed from the total phagotrophic community compared to $FLB_{FLS}$, also indicating that the community was not mainly adapted to graze on particle attached bacteria, as Starink et al. (1994a) found. Since they had to isolate organisms out of the sediment by an isopycnic centrifugation technique with nonlinear Percoll gradients (Starink et al. 1994b), it can be assumed that the sediment they investigated was much finer with higher portions of debris and organic material. It probably differed from the sediment I investigated, having greater portions of attached bacteria and a greater variety of feeding strategies of protists.

After the initial difference of MNF abundances with ingested $FLB_{nat/sed.}$ and ingested $FLB_{FLS}$, MNF abundances with either fluorescent tracer did not vary with incubation time. There was a trend of higher MNF abundances with ingested FLB after 16h, therefore this incubation time was chosen for all the following experiments.

In Experiment IV, there were no significant differences between the uses of $FLB_{nat/plank.}$ and $FLB_{Hal.}$ for MNF. In Newport Beach, there was a trend of greater abundances of MNF with ingested $FLB_{nat/plank.}$, which was an indication for me to use $FLB_{nat/plank.}$ in subsequent experiments. Mixotrophic contributions to the nanoflagellate communities investigated with these methods only present minimum estimates for several reasons. MNF and HNF, which have ingested FLB, do not necessarily still contain them at the time of fixation, because FLB could already have been egested again or digested without having ingested new FLB yet.

Furthermore, there is a possibility of feeding selectivity for or against the surrogates (relative to natural prey), which is based on size, phenotypic traits, motility or taste discrimination (Caron et al. 1999, Sanders et al. 2000, Matz et al. 2002). Size discrimination can either be positive or negative, but usually larger cells of 1-2µm are grazed at higher rates than smaller ones (0.5µm, Safi & Hall 1999). In my study, FLB were larger than natural bacteria due to the fact that DTAF binds to surface proteins of the cell, thus changing and

enlarging the bacterial structure. But motility and taste discrimination probably result in the selection of natural bacteria (Monger & Landry 1992), which may balance the size selectivity for larger FLB. However, bacterivorous flagellates showed significantly higher ingestion rates of motile compared to non-motile bacteria (González et al. 1993). According to Boenigk et al. (2002), flagellates discriminate against surrogate particles depending on their digestibility. They observed *Spumella* feeding on FLB and unstained bacteria and found similar ingestion rates of both, but FLB were egested after a vacuole passage time of only 3 minutes, whereas unstained bacteria stayed more than 20 minutes in the food vacuoles. After more than 3 minutes incubation time, the number of FLB observed in the food vacuoles reached a steady state, probably dependent on the concentration of FLB in the experiment and on vacuole passage time. Since fluorochromes themselves could not be found to be responsible for particle egestion (Premke & Arndt 2000), they assumed that the egestion of FLB is caused by DTAF, binding to cell surface proteins and probably blocking the flagellates' digestive enzymes. Food vacuole processing as well as ingestion, egestion or digestion rates are highly species-specific. Any labeling process probably changes the surface of prey particles, the structure, digestibility or something else. Working with natural communities consisting of a variety of heterotrophic and mixotrophic species, which all have species specific ingestion, egestion and digestion rates, different food vacuole passage times etc., makes it impossible to find an optimal method using labeled food surrogates. They will never suit the entire phagotrophic/mixotrophic community, since some species will always select against the tracer particles for one or the other reason.

In addition to these experimental artifacts, environmental factors, such as light or nutrient concentration may affect phagotrophy in mixotrophs, reducing FLB ingestion. The acquisition of nitrogen and phosphorus from particulate food, when dissolved nutrients are low, is one potential advantage of mixotrophy (Sanders 1991b, Nygaard & Tobiesen 1993). When dissolved nutrients are replete, phagotrophy may be reduced or ceased (Nygaard & Tobiesen 1993, Arenovski et al. 1995, Havskum & Riemann 1996). Furthermore, the individual physiological condition could affect estimates of abundances of mixotrophs, when, for instance, phagotrophic behavior is ceased during cell division (Boraas et al. 1992). The latter aspect can also influence feeding in heterotrophs among other factors. Only part of the HNF in my experiments contained ingested FLB, which is consistent with previous plankton studies (McManus & Okubo 1991, Šimek & Straskrabová 1992, Bratvold et al. 2000, Cleven & Weisse 2001).

In spite of all disadvantages, the use of surrogate labeled food tracers/particles such as FLB, as experimental evidence of feeding, is the only way to identify mixotrophic activity by algae in natural environments so far. Since there is no data available about mixotrophs of any natural benthic sediment communities, I can only discuss the patterns I found with data on mixotrophs in plankton communities. I decided to use the same kind of tracer method used in previous plankton studies (see Chapter 1) and found in the present study the most appropriate FLB method for the systems I wanted to investigate, using natural monodispersed benthic FLB with an experimental incubation time of 16h. In subsequent experiments described in the following chapters, I continued to use this method.

Previous plankton studies demonstrated that mixotrophic occurrence and abundances in marine ecosystems are highly variable in temporal and spatial scales. Sanders et al. (2000), for instance, reported percentages of MNF to the total phototrophic nanoflagellates in a range of <2% to 38% within only a few days at a coastal ocean site of Georges Bank (off the northeast USA), whereas they contributed from 4% to 39% to phagotrophic nanoplankton. Similarly varying contributions of mixotrophs to the total phototrophic nanoplankton (Arenovski et al. 1995, Havskum & Riemann 1996, Safi & Hall 1999) or to bacterivory and herbivory (Hall et al. 1993, Nygaard & Tobiesen 1993, Havskum & Riemann 1996, Havskum & Hansen 1997, Safi & Hall 1999) are known. Large proportions of MNF described above have important implications for algal nutrition, nutrient dynamics and food web interactions in planktonic ecosystems, which may also be present in benthic ecosystems. In order to understand mixotrophic dynamics in benthic systems and their potential importance at both trophic levels, it is necessary to investigate the same factors that play an important role for mixotrophic dynamics in plankton communities.

In the experiments described in this chapter, MNF responded to light limitation in dark incubations, i.e. part of the mixotrophic community switched from photosynthesis to phagotrophy. In the following Chapter 3, light and nutrient regimes are investigated as factors influencing the trophic mode of MNF and thus causing shifts in prey and nutrient dynamics, affecting the entire microbial community.

# Chapter 3

# The influence of light and nutrient conditions on the feeding strategy of benthic mixotrophic nanoflagellates

## 3.1. Introduction

Phagotrophy may be an important feature for mixotrophic flagellates. Grazing on bacteria may serve as an energy source, permitting flagellate growth and survival at low light conditions. It may also serve as an additional mechanism to obtain major nutrients (nitrogen N or phosphorus P), especially when concentrations of dissolved nutrients are low, or other specific growth factors or trace metals like iron (Sanders 1991a), Nygaard & Tobiesen 1993, Maranger et al. 1998). Experimental studies, investigating feeding habits of mixotrophic species under controlled laboratory conditions, demonstrated a considerable variation in the photosynthetic activity and particle ingestion among different mixotrophs (Sanders et al. 1990, Caron et al. 1993, Jones et al. 1993, Jones & Rees 1994ab, Keller et al. 1994). Most likely, phagotrophy fulfills different requirements for different phytoplankton species along a gradient of mixotrophic behavior, ranging from nearly pure phototrophy to nearly pure heterotrophy (Sanders et al. 1990, Jones 1994). Some mixotrophs act on one particular point of this range, whereas others are able to pursue different nutritional modes and move along this spectrum. Therefore, the relative importance of phagotrophy and photosynthesis within a species may vary in dependence of external factors, such as inorganic nutrients (Sanders et al. 1990, Jones et al. 1993). Phagotrophy may be reduced or ceased when dissolved nutrients are replete (Nygaard & Tobiesen 1993, Arenovski et al. 1995, Havskum & Riemann 1996). Conversely, nutrient limitation has been shown to stimulate mixotrophic grazing in some cases (Sibbald & Albright 1991, Rothhaupt 1996b). Bacteria have higher P/C ratios than algae (Fagerbakke et al. 1996) and are also more efficient at sequestering P at low concentrations (Currie & Kalff 1984, Bratbak & Thingstad 1985, Güde 1985). Therefore, under P-limited conditions, it would be a particularly efficient strategy for P-depleted algae to feed upon P-rich bacteria. In a number of marine studies, decreasing phagotrophic activity of mixotrophic algae with increasing water depth was attributed to the greater availability of dissolved nutrients at depth relative to surface waters (Nygaard & Tobiesen 1993, Arenovski et al. 1995, Havskum & Riemann 1996). Also in freshwater lakes, phagotrophy in mixotrophic algae was

linked to nutrient availability. The class Chrysophyceae contains many mixotrophic flagellates and often dominates phytoplankton biomass and production in oligotrophic and dystrophic lakes (e.g. Salonen & Jokinen 1988, Jansson et al. 1996). Besides inorganic nutrient concentrations, several environmental variables including prey density and light intensity can regulate phagotrophy in mixotrophic flagellates (Caron et al. 1990, 1993, Sanders et al. 1990, Sibbald & Albright 1991, Jones et al. 1993, Rothhaupt 1996a). For instance, Bird & Kalff (1986) found mixotrophic flagellates to be abundant in metalimnic phytoplankton maxima and attributed their high abundances to low light intensities limiting photosynthesis. Feeding responses to these factors appear to be highly variable from species to species (Raven 1997). Natural mixotrophic communities comprise a multitude of species and it is most likely that several factors are simultaneously involved in the regulation of grazing activity.

Studies on mixotrophs are restricted to plankton communities so far (see Chapter 2). Most studies investigated factors regulating mixotrophy in particular species, but few attempts have been made to identify those regulating factors in natural communities (Isaksson et al. 1999). In natural aquatic sediments, there is no information about the importance of mixotrophs in the flow of energy at all. In the present study the quantitative importance of mixotrophic flagellates as bacterial grazers and primary producers in natural microbial communities in coastal marine sandy sediments is investigated in dependence of nutrient and light availability. MNF responded to light limitation (see Chapter 2), i.e. part of the mixotrophic community was able to switch from photosynthesis to phagotrophy. This was shown by increasing abundances of MNF with ingested FLB in dark incubations. Here, three experiments are presented, where benthic mixotrophic flagellates in coastal marine sediments were incubated under different nutrient and light conditions. Nutrient depleted and *in situ* sediment was incubated in a climate chamber in the dark (Experiment I), in the light (Experiment II) and in both light and dark (Experiment III), to compare how these factors influence phagotrophic activity in mixotrophs, and how they interact. Sediments were incubated for 3-7 days and FLB-grazing experiments were conducted on the first and the last day of sediment incubation. With the experiment on day 1, I obtained abundances of mixotrophic nanoflagellates and their responses to different short-term treatments in the initial flagellate community; in the second experiment I investigated possible community changes after several days of sediment incubation due to different treatments.

Beside the investigation of mixotrophic abundances in the coastal marine sediment of Falckenstein Beach in the Baltic Sea in two different seasons, this study provides information

about the ability of benthic mixotrophs to respond to changing light and nutrient conditions and consequently about the importance of mixotrophic dynamics within the energy flow in the sediment.

## 3.2. Material and Methods

### 3.2.1. Experiments

Three long-term grazing experiments were conducted with sediment from Falckenstein Beach, Kiel Fjord, Western Baltic Sea (10° 11' 40''E, 54° 24' 23''N) in February (I and II) and October (III) 2001. It was the same study site already characterized in Chapter 2. Autumn and winter conditions were chosen to compare results with findings in Chapter 2.

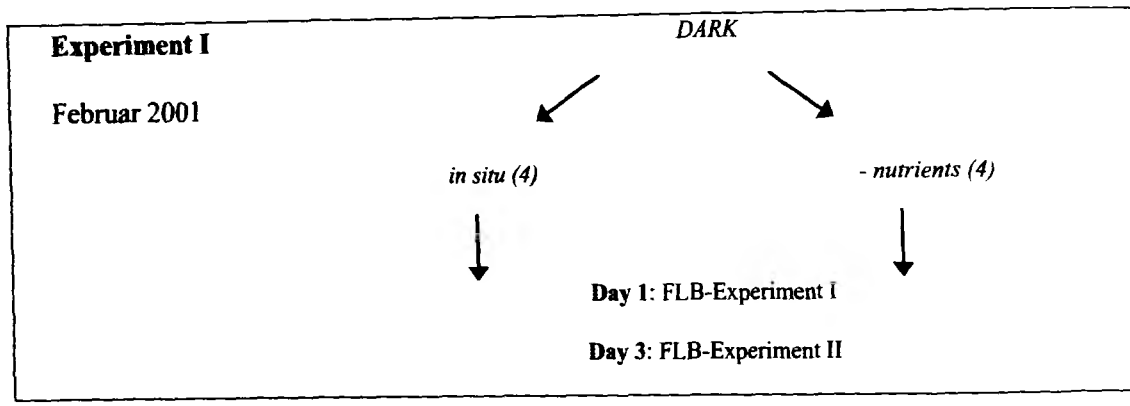
| | **Experiment I** | | | **Experiment II** | | | **Experiment III** | | |
|---|---|---|---|---|---|---|---|---|---|
| **samples** | BS*is*<br>*RS*-n | d1*is*<br>*d1*-n | d3*is*<br>*d3*-n | BS*is*<br>*RS*-n | d1*is*<br>*d1*-n | d7*is*<br>*d7*-n | BS*is*<br>*RS*-n | d1*is*<br>*d1*-n | d3*is*<br>*d3*-n |
| **soluble N (sum of $NO_3^{2-}$, $NH^{4+}$)** | *22.74*<br>1.38 | *15.33*<br>5.47 | *29.02*<br>15.57 | *22.74*<br>1.38 | *16.04*<br>4.37 | *13.63*<br>6.80 | *14.09*<br>1.76 | nd | nd |
| **soluble P** | *1.00*<br>0.07 | *1.27*<br>0.25 | *0.75*<br>0.58 | *1.00*<br>0.07 | *0.98*<br>0.31 | *0.65*<br>0.30 | *1.46*<br>0.01 | nd | nd |
| **N : P** | *22.74*<br>19.71 | *12.07*<br>21.88 | *38.69*<br>26.85 | *22.74*<br>19.71 | *16.37*<br>14.1 | *20.97*<br>22.67 | *9.65*<br>176.0 | nd | nd |

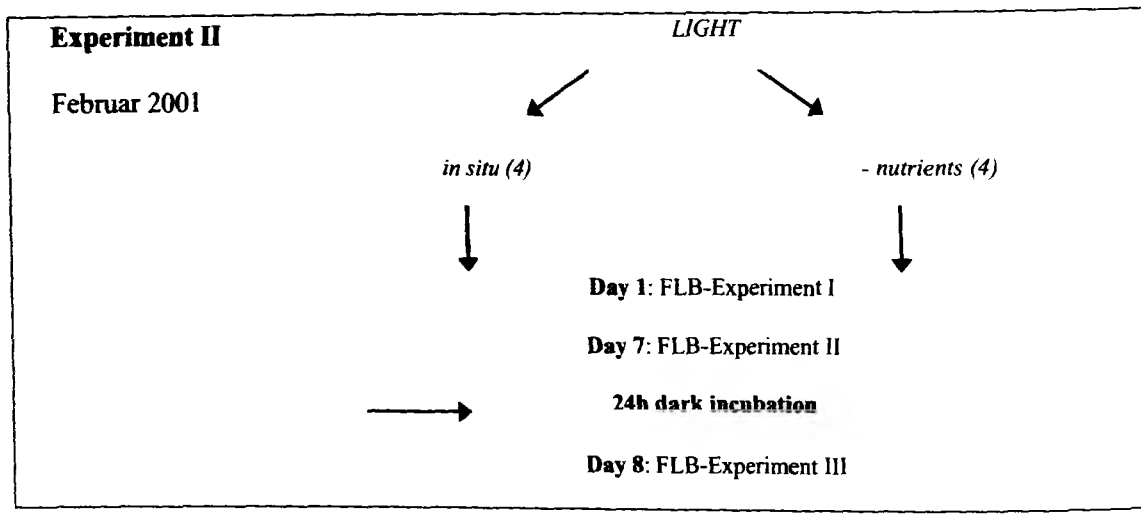
**Table 3.1.** Dissolved nitrogen N and phosphorus P in nutrient depleted (-n) and *in situ* (*is, values in italics*) sediments of all experiments I to III. BS and RS present values in the incubation water of the Baltic Sea (BS) and the Red Sea (RS), respectively; d1, d3 and d7 present different sampling days of the experiments, where FLB experiments were conducted. In Experiment III, only values of the incubation water (BS and RS) are presented, since measurements of samples of the overlying water during Experiment III failed (nd = non determined).

Sediment of the surface layer of Falckenstein Beach was collected 2m behind the shoreline in shallow water at a water depth of 0.3 - 0.5m. In the laboratory, part of the sediment was filled into water basins (acrylic glass, 20cm x 15cm x 15cm) and covered with 5cm of *in situ* water of Kiel Fjord. The other part was filled into dialyzing tubes (Reichelt Chemietechnik, Thomapor®-Standard RCT® 88-09, width=70mm, inner diameter=55mm, volume=13.85), and incubated in extremely nutrient poor, oligotrophic water from the Red Sea (Table 3.1.), which was adapted to the salinity of Kiel Fjord before (15psu). This was done in order to "wash out" inorganic nutrients out of the sediment to induce nutrient depletion. Nutrient poor water was exchanged every two hours to keep the nutrient diffusion going. After 6h, the

nutrient depleted sediment (see Table 3.1.) was also transferred from the dialyzing tubes into water basins, and covered with 5cm of nutrient poor water. Mixotrophs exhibited higher phagotrophic activities in dark incubations in previous experiments (see Chapter 2). In order to investigate if phagotrophic activity would further increase under nutrient depletion, nutrient depleted and *in situ* sediment was incubated in the dark for three days in Experiment I. Four replicate basins were incubated per treatment, i.e. *in situ* sediment and nutrient depleted sediment (-nutr.), resulting in a total of 8 basins (Table 3.2.). The dark incubations were covered with black cloth. All basins were incubated in a climate chamber at 18°C with a light/dark cycle of 16:8 hours at a light intensity of 60µE $m^{-2}s^{-1}$ (LICOR Quantum Photometer LI-185B). After 24h (day 1) and 72h (day 3), sediment cores were taken out of the 4 replicate basins, respectively, and FLB grazing experiments were conducted (as described in Chapter 2) to obtain abundances of MNF. Four replicate sediment cores were taken per treatment for t0 and t16 in every experiment. Sampling had to be destructive, because it was not possible to take quantitative subsamples out of the sediment incubations. Hence, one set of replicates for each treatment had to be taken and incubated for every census of each FLB grazing experiment, resulting in a total of two sets. In this experiment, the nanoflagellate community consisted mainly of heterotrophs with minor contributions of MNF. Therefore, I conducted a second experiment with manipulated sediment, where nutrient depleted and *in situ* sediment was subjected to a light/dark cycle of 16:8 hours for 7 days (Table 3.2.). With this light/dark cycle, a day/night rhythm was simulated, but with the light phase being much longer than the natural light phase during the day in February. This treatment (called "light" in the following) favored photosynthesis and with that phototrophs and potentially mixotrophic flagellates. Under the assumption that more phototrophs with the potential to be mixotrophic would develop during 7 days of light incubation, nutrient depleted and *in situ* sediment afterwards was incubated in the dark for 24h, to induce higher phagotrophic activities in mixotrophs. FLB grazing would make it possible to identify them as mixotroph and to investigate which part of the mixotrophic community was able to show facultative heterotrophy when photosynthesis was light limited.

FLB grazing experiments in Experiment II were conducted 24h (day 1) after sediment incubation, after 7 days of light incubation and after the 24h dark incubation (day 8) (Table 3.2.) in the same way as described in Chapter 2. Again, 4 replicate sediment cores were taken out of the 4 replicate basins per treatment, for t0 and t16 sampling in the FLB experiment, resulting in a total of 16 cores per grazing experiment (2 cores per basin).



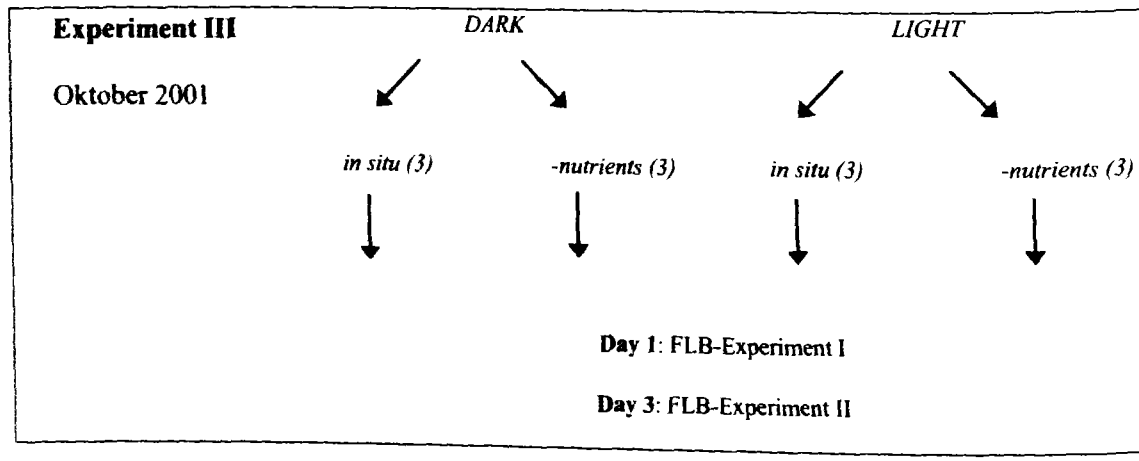




**Table 3.2.** Experimental design of Experiment I, II and III. The basins incubated with different sediment treatments are written in italics with the number of replicates in parentheses; sampling times and further processing are presented beneath.

In Experiment III, light and nutrient conditions were combined in a factorial design with nutrient depleted and *in situ* sediment, both incubated in light and dark. With this experiment,

I was able to obtain possible interrelations of the two factors, affecting the mixotrophic community. All treatments were sampled for FLB grazing experiments after 24h (day 1) and 72h (day 3) sediment incubation. In this experiment, only 3 replicate basins per treatment were incubated due to an incident damaging basins, i.e. *in situ* sediment and nutrient depleted sediment (-nutr.) were incubated in light and dark, respectively, resulting in a total of 12 basins (Table 3.2.). All dark incubations were covered with black cloth and then all basins were incubated in a climate chamber at 18°C with a light/dark cycle of 16:8 hours. FLB grazing experiments were conducted on day 1 and day 3 after sediment incubation, taking 4 sediment cores for each census (t0 and t16) (Table 3.2.) to extrude them and to conduct FLB grazing experiments.

### 3.2.2. FLB preparation

Sediment from the sampling site at Falckenstein Beach was collected one week before conducting the respective experiments. Natural bacteria were isolated from this sediment and monodispersed natural benthic FLB were prepared as described in Chapter 2. With this procedure, I strived towards getting labeled bacteria that matched the natural prey spectrum of nanoflagellates in this system at the time of the experiment to the greatest possible extent.

### 3.2.3. Sampling procedure

In all experiments, sediment subsamples for live counts (see below) were taken out of the basins for every treatment for every FLB grazing experiment with a cut-off syringe. These samples were diluted with SSW (1:10), stored in the climate chamber at 18°C and processed within 2h after sampling.

Nutrients were analyzed in *in situ* water of Kiel Fjord, which was added to the *in situ* sediment and in the oligotrophic Red Sea water, whose salinity was adapted to Kiel Fjord water. After the *in situ* and the dialyzed sediment had been incubated in the climate chamber for 3 to 7 days, the overlying water column directly above the sediment was sampled for nutrient analysis. Subsamples of water were filtered through acid washed Whatman GF/F filters for nutrient analysis. Dissolved nutrients (Ammonia ($NH_4$-N), nitrate ($NO_3$-N) and dissolved inorganic phosphorus (DIP)) concentrations were measured using a SKALAR SCANPLUS SYSTEM autoanalyser.

*Experiment I*

24h after sediment incubation in the dark (day 1), the first FLB experiment was conducted (Table 3.2.). The acrylic sediment cores already described in Chapter 2 were used. Two cores were taken out of every replicate basin per treatment for t0 and t16 sampling of the FLB experiment, resulting in a total of eight cores (Table 3.2.). FLB experiments were conducted as described in Chapter 2 (see Fig. 2.1.). The first 3mm of the sediment cores (1.5$cm^3$ +/- 0,4 $cm^3$) were extruded and transferred in wells of tissue culture plates (Renner, Tissue Culture Test Plates, 6 wells/plate, radius/well = 3.45 cm, volume/well = 15.53 $cm^3$), which contained 2 ml of sterile filtered seawater (SSW). The sediment density was determined and each sediment slice was weighed in order to calculate accurate sediment volumes for each sample afterwards. FLB were added, approximating 25% of the natural bacterial abundances, and t0 samples were fixed with ice cold glutardialdehyde at a final concentration of 1.5%. Dark incubations were covered with aluminum foil and all t16 samples were incubated in the climate chamber at 18°C. After 16h, the second set of replicates was fixed and all samples preserved at 4°C until further processing. After 72h (day 3), the second FLB experiment was conducted in the same way as the foregoing experiment.

*Experiment II*

Nutrient depleted and *in situ* sediment (4 replicate basins per treatment) was subjected to a light/dark cycle (see above) in a climate chamber at 18°C and a light intensity of 60µE $m^{-2}s^{-1}$. 24h after sediment incubation (day 1), the first FLB-experiment was conducted and on day 7 the second one in the same way as described for Experiment I. After that, the sediment was covered with black tissue and was incubated in the dark. After 24h (day 8), the third FLB experiment was conducted. Again, fixed samples were preserved at 4°C until further processing.

*Experiment III*

Nutrient depleted and *in situ* sediment was incubated both in the dark and light with 3 basins per treatment under the same experimental conditions as in Experiment I and II (Table 3.2.). After 24h (day 1) and 72h (day 3) of sediment incubation, 8 sediment cores for t0 and t16 sampling were taken randomly out of the 3 basins, i.e. the 4 replicates processed in the FLB experiments were taken out of only 3 replicate basins. This deficiency of the experimental design (slight pseudoreplication, 2 cores were taken out of the same basin) was accounted for in the statistical analysis (see below). FLB grazing experiments were conducted by processing the 4 sediment cores in the same way as described in Experiment I and II.

### 3.2.4. Sample processing

Samples were processed in the same way as described in Chapter 2 (see Fig. 2.1.). The sediment was diluted and a subsample was fixed with glutardialdehyde (final concentration of 1.5%). A subsample was collected on a 0.2µm polycarbonate filter (Nucleopore Track-Etch Membrane, PC MB 25mm 0.2µm) and stained with DAPI (4', 6-Diamidino-2-phenylindol) for 5min at a final concentration of 5 µg/ml to stain bacteria and nuclei of protists (Porter & Feig 1980, Sherr & Sherr 1993). Filters were mounted on slides, sealed with paraffin wax and stored at -20°C until the microscopical analysis took place.

### 3.2.5. Counting

Filters for the determination of flagellate, bacterial and FLB abundances were counted with an epifluorescence microscope (Leica/Leitz DMRB) at 1000x magnification in the same way as in previous experiments described in Chapter 2.

In addition to the general quantifications, diluted sediment subsamples (1:10) were analyzed in order to classify flagellates into major systematic groups, using the live counting technique (e.g. Gasol 1993, Dietrich & Arndt 2000). Diluted sediment subsamples were thoroughly mixed and two to three aliquots of 5-20µl were counted per sampling day (see Table 3.1.) on a slide under an upright phase contrast microscope at 400x magnification (Leitz Dialux 20, Wetzlar Germany). For flagellates, the live counting method is helpful in determining the taxonomic structure of the community since size and form of the body as well as the movement of the flagella are used in species determination (e.g. Foissner 1991, Patterson & Larson 1991). In live counts, flagellates were classified into the following groups: Kinetoplasta, Cryptomonada, Chrysomonadea, Dinoflagellata, Cercomonadida, Thaumatomastigida and Apusomonadida. Whereas no phototrophic forms exist in the phylum Kinetoplasta, the subphylum Cryptomonadeda consists mostly of phototrophic forms. The class Chrysomonadea and the subphylum Dinoflagellata consist of both heterotrophic and phototrophic forms. Cercomonadida, Thaumatomastigida and Apusmonadida all belong to the group of *Incertae sedis*, consisting of species, which can not be subordinated with certainty into the taxonomic system established so far (Hausmann & Hülsmann 1996), Cercomonadida consisting of phototrophs and heterotrophs and Apusomonadida and Thaumatomastigida of heterotrophs.

### 3.2.6. Statistical Analysis

In order to test for differences between nutrient depleted and *in situ* sediment in Experiment I, a repeated measurement ANOVA was conducted with nutrient treatment (*in situ*/-nutrients) as independent factor between subjects and time (abundances of MNF on day 1 and day 3) as independent factor within subject. Normal distribution and homogeneity of variances were tested with a Chi-Square-Test and with a Bartlett-Chi-Square-Test, respectively. The same ANOVA was conducted for HNF, PNF and bacteria in Experiment I. In Experiment II, a repeated measurement ANOVA was also conducted for MNF, PNF, HNF and bacteria, respectively, with nutrient treatment (between subjects) and time (within subjects, abundances on day 1, 7 and 8) as independent factors. In Experiment III, a repeated measurement ANOVA was conducted with 2 independent factors between subjects, light (light/dark) and nutrients (*in situ*/-nutrients), and time (abundances on day 1 and day 3) as factor within subjects, also on abundances of MNF, PNF, HNF and bacteria on day 1 and day 3. Since two replicate cores were taken out of the same basin, and could therefore not be regarded as (statistically) independent replicates, I recalculated the degrees of freedom (df) for the error terms using 3 replicates instead of 4.

## 3.3. Results

In all experiments described here, the nanoflagellate communities were clearly dominated by heterotrophs at all time points. In Exp. I and II, they contributed 70% to 80% to the total nanoflagellates (Fig. 3.1.). Phototrophic contributions (PNF) counted about 20% whereas mixotrophs (MNF) were only presented with 1.5% to 2.8% of the total nanoflagellate community (Fig. 3.1.). Only in Experiment III, initially dominant HNF contributions decreased down to 55% and PNF and MNF contributions increased to 35-40% and 6-7%, respectively (Fig. 3.1.).

Nutrient concentrations in nutrient depleted basins were 3-4 times lower than in *in situ* basins on day 1 after sediment incubation in Experiment I and II (Table 3.1.). Nutrients increased again after 3 and 7 days sediment incubation, respectively, but were still lower than in *in situ* basins (Table 3.1.).

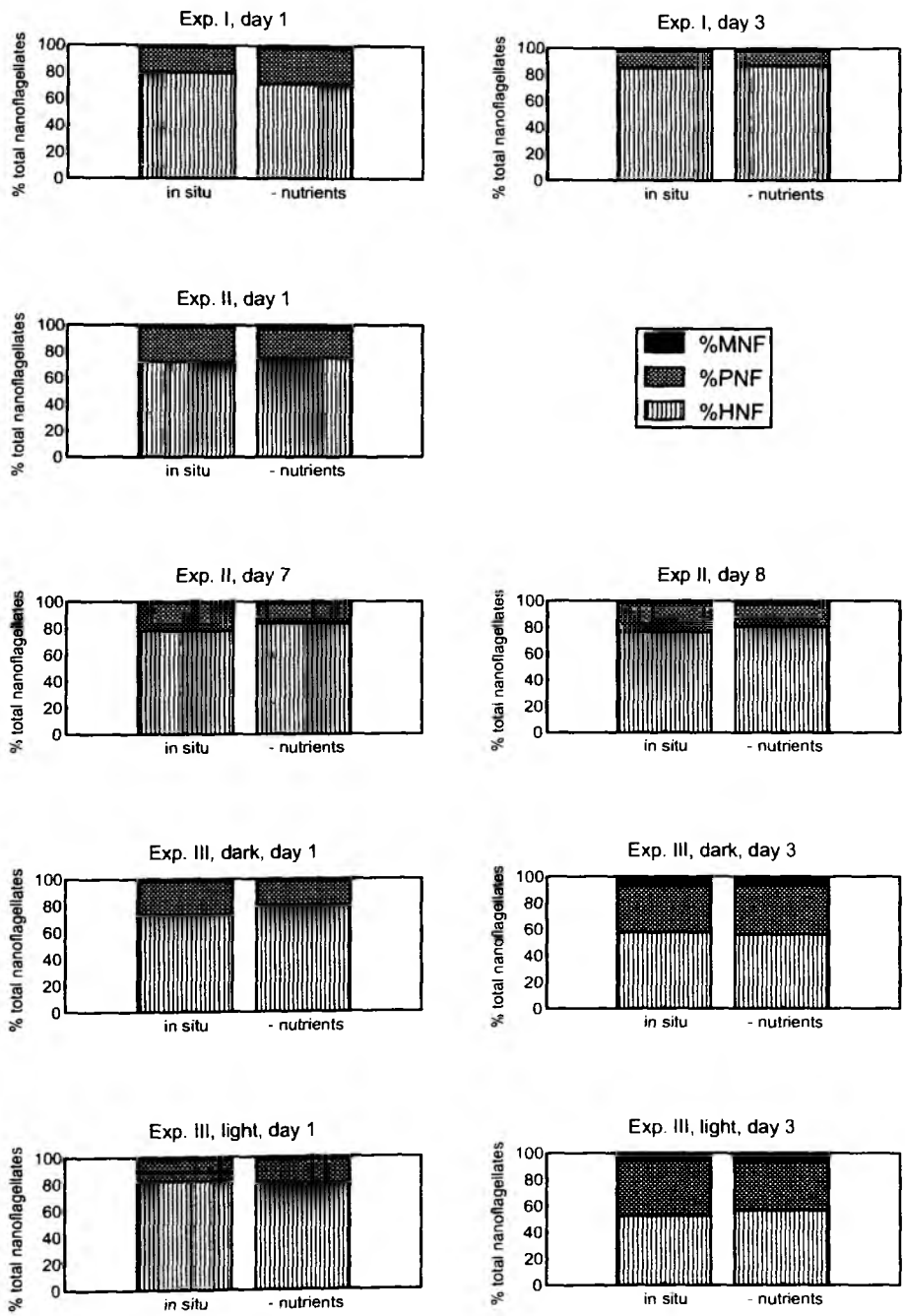



**Fig. 3.1.** Relative abundances of heterotrophic (HNF), phototrophic (PNF) and mixotrophic (MNF) nanoflagellates as percentage of the total nanoflagellates in Experiments I to III in all experimental treatments and censuses.

## Experiment I

*Abundances*

Total HNF reached abundances of $5x10^4$ - $1x10^5$/cm³ sediment, PNF of $1x10^4$/cm³, MNF of $1x10^3$/cm³ and bacteria of $5x10^7$/cm³ (Fig. 3.2. and 3.3.). Abundances of the total HNF varied significantly with time (Table 3.4). The interaction between time and treatment was significant, too, indicating that the nutrient treatment had disparate effects on different days. On day 1, abundances of HNF did not differ in nutrient depleted and *in situ* sediment. On day 3, HNF abundances in the nutrient depleted sediment increased and exceeded abundances on day 1 and in *in situ* sediment (Fig. 3.2.), indicating that the nutrient treatment affected HNF abundances increasingly with time. Abundances of PNF and identified MNF with ingested FLB were neither affected by different nutrient treatments nor by incubation time (Table 3.4., Fig. 3.2.). However, there was a trend of more MNF with ingested FLB in the nutrient depleted sediment compared to *in situ* sediment on day 3 (Fig. 3.4.). Abundances of bacteria did not differ significantly with treatment or time (Table 3.4.); there was a trend of more bacteria in *in situ* sediment than in nutrient depleted sediment on day 1, whereas this trend was reversed on day 3 (Fig. 3.3.).

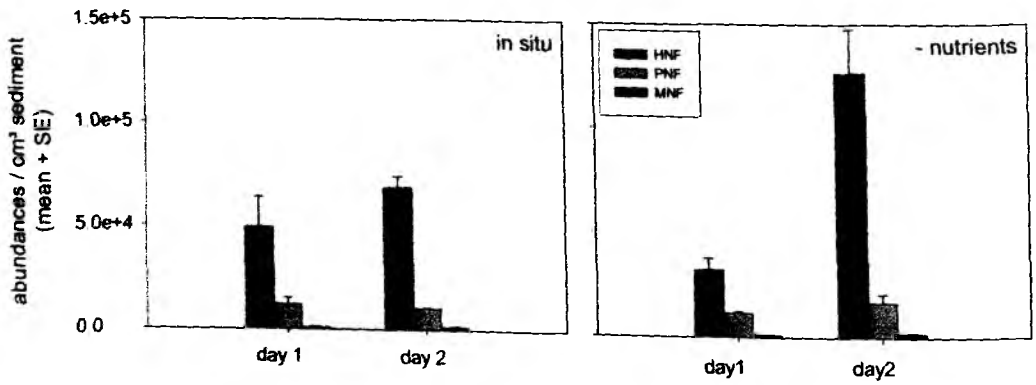



**Fig. 3.2.** Microbial community structure in nutrient depleted and in situ sediment n day 1 and 3 in Experiment I. Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF) and mixotrophic nanoflagellates (MNF) per cm³ sediment.

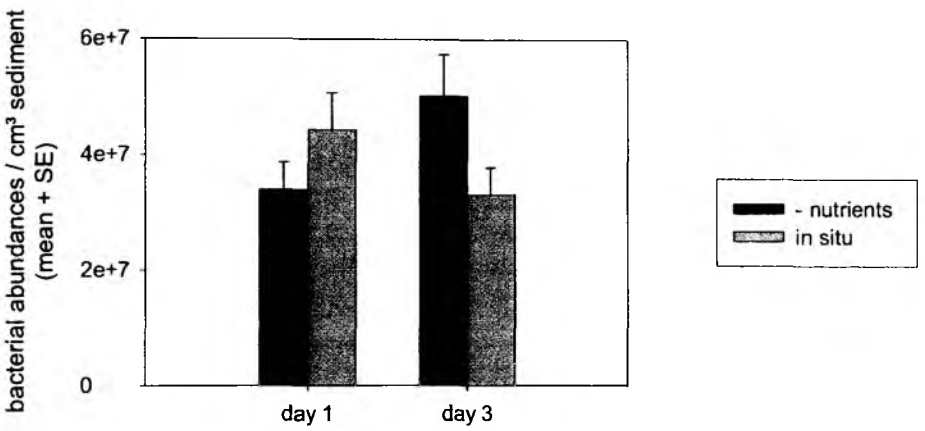



**Fig. 3.3**. Absolute abundances of bacteria per $cm^3$ sediment in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment I.

| Experiment | %MNF/NF in situ | %MNF/NF - nutrients | %HNF+FLB/ total HNF in situ | %HNF+FLB/ total HNF - nutrients |
|---|---|---|---|---|
| **I day 1 *dark*** | 2.3 ± 0.9 | 2.8 ± 0.4 | 8.2 ± 1.7 | 15.5 ± 2.6 |
| **I day 3 *dark*** | 1.6 ± 0.4 | 1.3 ± 0.1 | 14.2 ± 1.2 | 11.7 ± 0.8 |
| **II day 1 *light*** | 1.1 ± 0.7 | 3.8 ± 0.5 | 12.6 ± 4.5 | 14.3 ± 2.7 |
| **II day 7 *light*** | 1.1 ± 0.4 | 1.2 ± 0.3 | 11.3 ± 1.8 | 10.4 ± 1.1 |
| **II day 8 *dark*** | 2.0 ± 0.6 | 2.2 ± 0.4 | 16.0 ± 1.1 | 10.2 ± 1.8 |
| **III day 1 *dark*** | 2.4 ± 0.9 | 2.1 ± 0.5 | 14.2 ± 0.7 | 12.8 ± 0.8 |
| **III day 1 *light*** | 1.3 ± 0.6 | 1.2 ± 0.3 | 9.5 ± 1.1 | 7.6 ± 0.7 |
| **III day 3 *dark*** | 7.1 ± 1.0 | 6.2 ± 1.0 | 25.6 ± 2.1 | 26.9 ± 2.4 |
| **III day 3 *light*** | 6.3 ± 1.3 | 7.3 ± 0.6 | 26.1 ± 3.3 | 23.4 ± 2.8 |

**Table 3.3**. Relative abundances of mixotrophic nanoflagellates as % of the total nanoflagellates (%MNF/NF) and heterotrophic nanoflagellates with ingested FLB as % of the total heterotrophic nanoflagellates (%HNF+FLB/ total HNF) for all Experiments I – III. Values in Experiment I and II refer to dark incubations. Values in Experiment III are averaged over all incubation times. Values in Experiment IV refer to the use of natural FLB. Values present mean (n=4) ± standard error.

| Factor | total HNF | PNF | MNF | Bacteria |
|---|---|---|---|---|
| **df** | 6 | 6 | 6 | 6 |
| **nutrients (1)** | 2.179<br>(0.190) | 1.218<br>(0.312) | 0.757<br>(0.418) | 0.890<br>(0.382) |
| **time (1)** | 22.245<br>(0.003) | 0.410<br>(0.546) | 1.580<br>(0.255) | 0.101<br>(0.761) |
| **nutrients x time (1)** | 11.056<br>**(0.016)** | 1.752<br>(0.234) | 0.856<br>(0.391) | 3.917<br>*(0.095)* |

(B)

| Factor | total HNF | PNF | MNF | Bacteria |
|---|---|---|---|---|
| **df** | 6 (between)<br>12 (within) | 6 (between)<br>12 (within) | 6 (between)<br>12 (within) | 6 (between)<br>12 (within) |
| **nutrients (1)** | 0.247<br>(0.637) | 3.479<br>(0.111) | 1.163<br>(0.322) | 6.725<br>**(0.041)** |
| **time (1)** | 12.756<br>**(0.001)** | 4.003<br>**(0.047)** | 3.889<br>**(0.050)** | 10.259<br>**(0.003)** |
| **nutrients x time (1)** | 0.667<br>(0.531) | 0.076<br>(0.927) | 0.596<br>(0.567) | 2.581<br>(0.117) |

**Table 3.4.** Results of repeated measurement ANOVA on abundances of total heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophs (MNF) and bacteria, with nutrient treatment as between-group factor and time as within-group factor (abundances in different sampling days). The table gives the F-ratios (with significance levels in parentheses) for the main factors for Experiments I to III. The degrees of freedom for the effect terms are given in parentheses for each effect, for the error term in the row on top of the analysis. Effects significant at $p<0.05$ are printed in bold, trends with $p<0.1$ are printed in italics. (A) Experiment I. The results for PNF should be considered with care since a violation of variance homogeneity was detected (PNF (day 1): Bartlett's $\chi^2=4.422$, $p=0.036$; PNF (day 3): Bartlett's $\chi^2=10.702$, $p=0.001$). (B) Experiment II. The results for PNF on day 1 should be considered with care since a violation of variance homogeneity was detected (Bartlett's $\chi^2=4.957$, $p=0.026$).

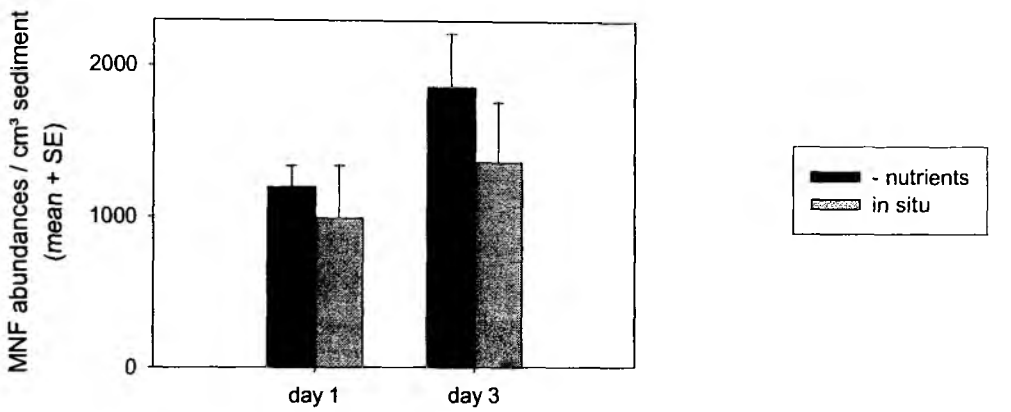



**Fig. 3.4**. Absolute abundances of mixotrophic nanoflagellates per $cm^3$ sediment in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment I.

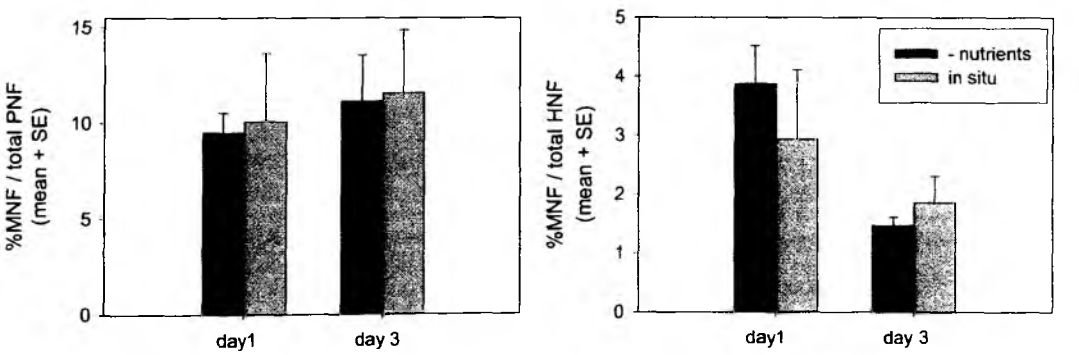



**Fig. 3.5**. Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic NF and the total heterotrophic NF (HNF+MNF) in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment I. Note the different scalings on the y-axes.

*Contributions*

Identified MNF contributed around 10% to the total PNF and maximum portions of 4% to the total HNF on day 1, and only about 2% on day 3 (Fig. 3.5.). Grazing activity of HNF, i.e. HNF with ingested FLB as percentage of the total nanoflagellates, ranged from 8% to 15% (Table 3.3.).

*Taxonomic composition*

Community composition in different nutrient treatments did not differ markedly on day 1 (Fig. 3.6.). Abundances of chrysomonads slightly increased during 3 days of dark incubation

in *in situ* sediments, but drastically increased in nutrient depleted sediments from approximately 20% on day 1 to more than 50% on day 3 (Fig. 3.6.). Dinoflagellates contributed only minor portions to the flagellate community. Thaumatomastigida decreased noticeably in the nutrient depleted sediment compared to the *in situ* one and Euglenida decreased from day 1 to day 3.

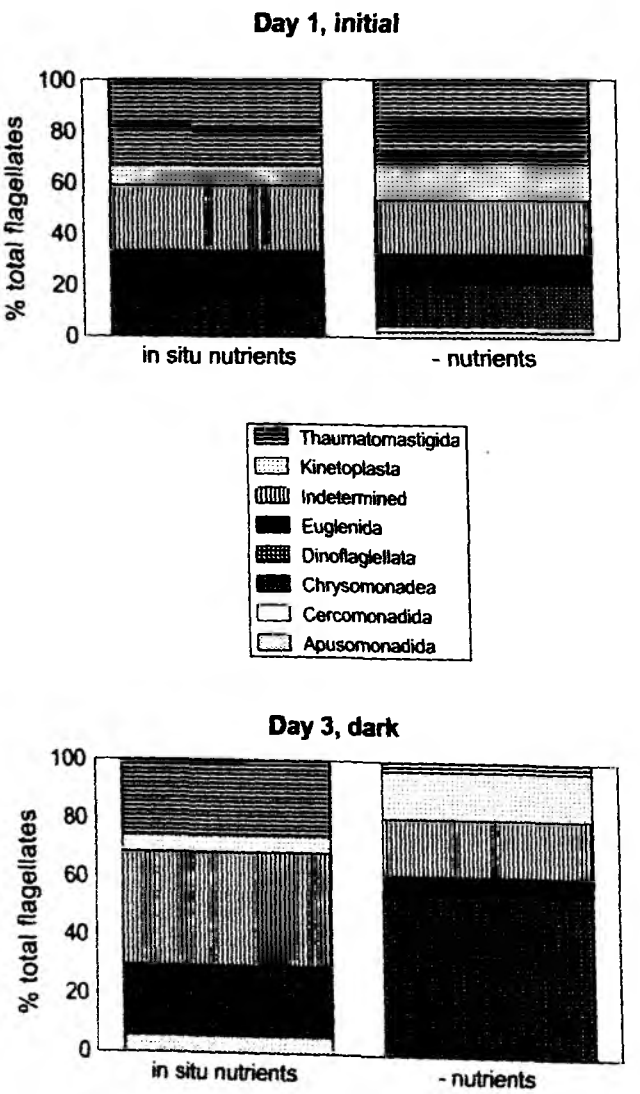



**Fig. 3.6.** Species composition in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment I. Relative abundances of different taxonomic groups are calculated as % of the total flagellates.

## Experiment II

*Abundances*

Absolute abundances of HNF, PNF, MNF and bacteria were in the same range as in Experiment I (Fig. 3.7. and 3.8.). Their abundances increased significantly with time from day 1 to day 8 (Table 3.4., Figs. 3.7., 3.8. and 3.9.). Bacteria showed significantly higher abundances in nutrient depleted sediments (on day 7 and day 8) than in *in situ* sediments (Table 3.4., Fig. 3.8.). There was also a trend of more MNF with ingested FLB in nutrient depleted sediments compared to *in situ* sediments on day 1. This effect leveled off until day 7 and was not evident anymore on day 8 after 24h incubation in the dark (Fig. 3.9.).

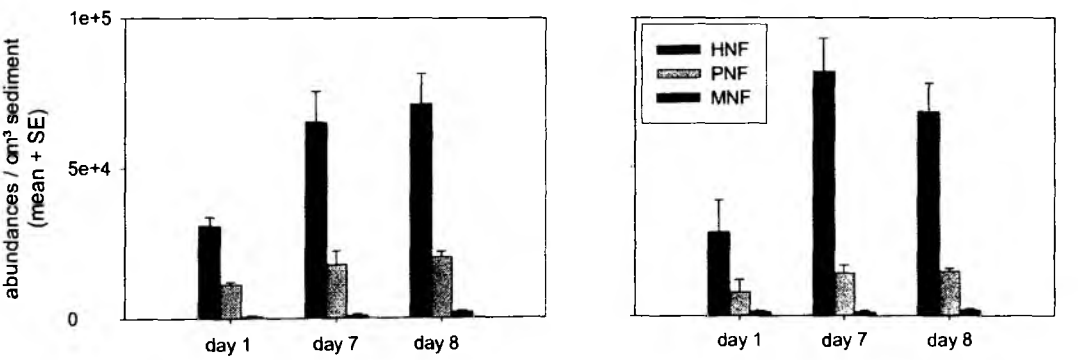



**Fig. 3.7.** Microbial community structure in nutrient depleted and in situ sediment on day 1, 7 and 8 in Experiment II. Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF) and mixotrophic nanoflagellates (MNF) per $cm^3$ sediment.

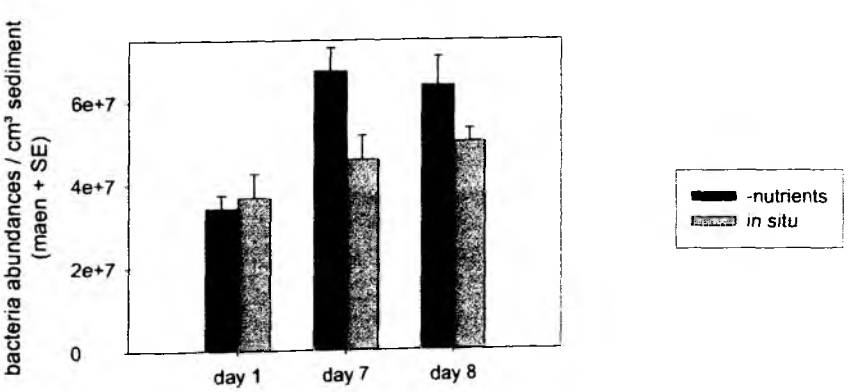



**Fig. 3.8.** Absolute abundances of bacteria per $cm^3$ sediment in nutrient depleted and in situ sediment on day 1, 7 and 8 of Experiment II.

*Contributions*

MNF contributed about 20% to the total PNF in nutrient depleted sediment on day 1, whereas contributions ranged only from 5% to 10% in *in situ* sediment and on day 7 and 8 (Fig. 3.10.). MNF contributions to the total HNF ranged between 1% and 5% (Fig. 3.10.). Grazing activity of HNF, i.e. HNF with ingested FLB as percentage of the total nanoflagellates, ranged from 10% to 16% (Table 3.3.).

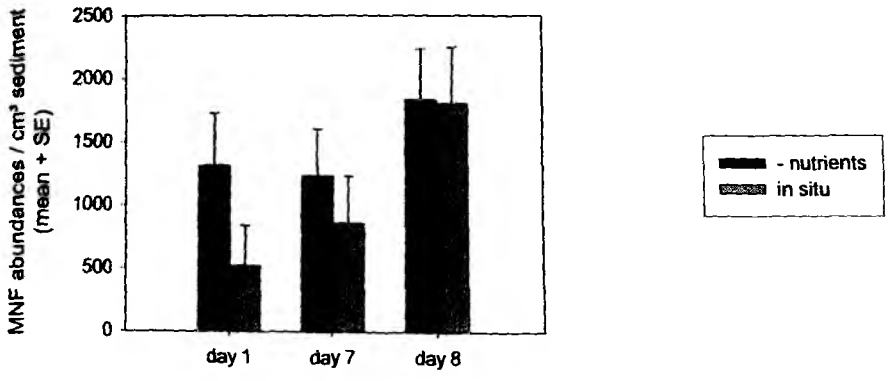



**Fig. 3.9.** Absolute abundances of mixotrophic nanoflagellates (MNF) per cm³ sediment in nutrient depleted and in situ sediment on day 1, 7 and 8 of Experiment II.

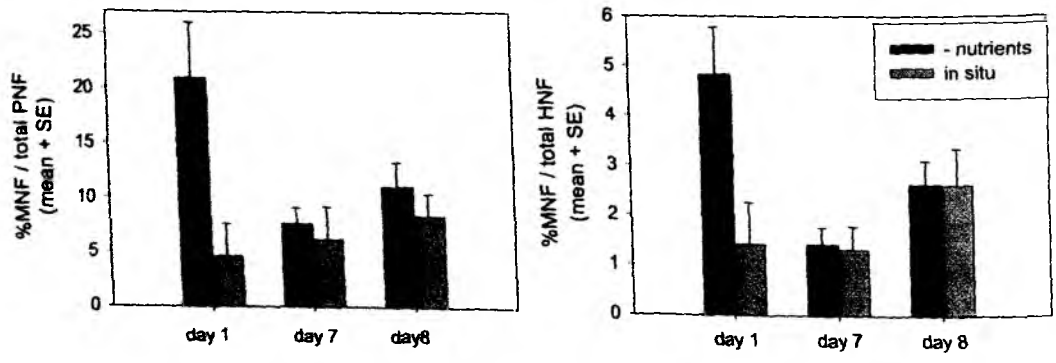



**Fig. 3.10.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates (PNF) and the total heterotrophic nanoflagellates (HNF) in nutrient depleted and in situ sediment on day 1, 7 and 8 of Experiment II.

*Taxonomic composition*

Flagellate community composition hardly differed in different nutrient treatments on day 1, 7 and 8, respectively (Fig. 3.11). Again dinoflagellates did not contribute considerably to the flagellate community, but chrysomonads increased from less than 20% to almost 60% on day 7 and even 70% on day 8 in both nutrient depleted and *in situ* sediments.

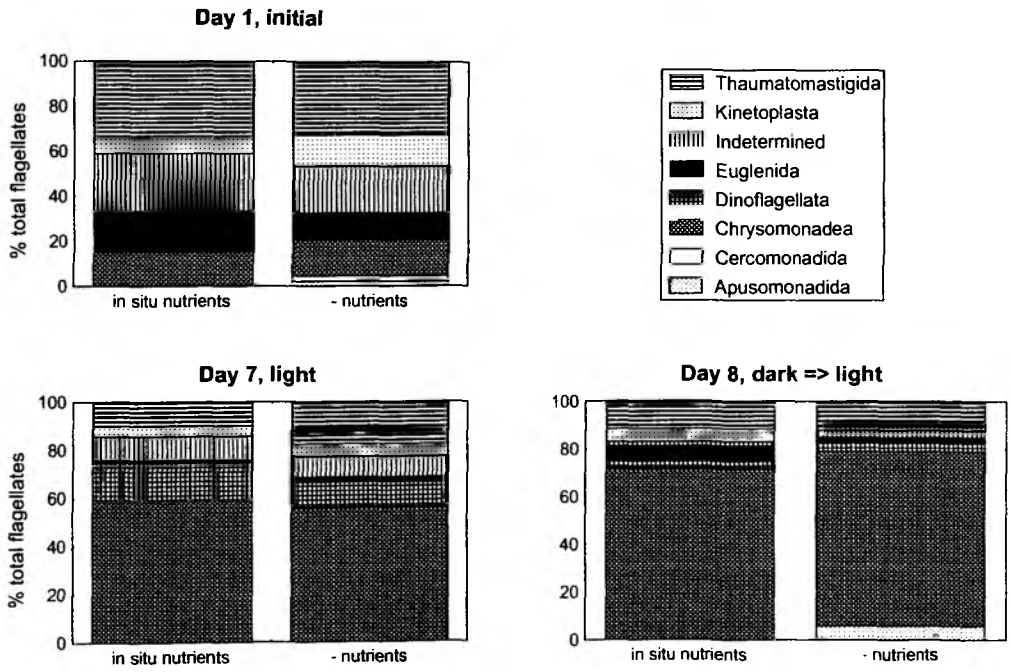



**Fig. 3.11**. Species composition in nutrient depleted and in situ sediment on day 1, 7 and 8 of Experiment II. Relative abundances of different taxonomic groups are calculated as % of the total flagellates.

## Experiment III

*Abundances*

Absolute abundances of the microbial community were approximately one decimal power higher than in the previous experiments (Fig. 3.12., 3.13. and 3.14.). Abundances of HNF and bacteria did not vary with either time or treatments (Table 3.5., Fig. 3.12. and 3.13.). Abundances of PNF and MNF significantly increased from day 1 to day 3, but hardly varied in different light or nutrient treatments (Table 3.5., Fig. 3.12. and 3.14.). The interaction between all three factors (light/dark, -nutrient/*in situ* and time) was significant for PNF abundances, indicating that treatment effects were dependent on one another and on time.

*Contributions*

MNF contributed between 10% (day 1) and 20% (day 3) to the total PNF, whereas they only contributed 2-3% to the total HNF on day 1 and 10-12% on day 3 (Fig. 3.15.). Grazing activity of HNF, i.e. HNF with ingested FLB as percentage of the total nanoflagellates, ranged from 8% to 26% (Table 3.3.).

| Factor | total HNF | PNF | MNF | Bacteria |
|---|---|---|---|---|
| **df** | 8 | 8 | 8 | 8 |
| **nutrients (1)** | 0.175 (> 0.25) | 0.300 (> 0.25) | 0.163 (> 0.25) | 1.228 (> 0.25) |
| **light/dark (1)** | 0.044 (> 0.25) | 0.001 (> 0.25) | 0.119 (> 0.25) | 1.376 (> 0.25) |
| **time (1)** | 0.589 (> 0.25) | 57.581 **(< 0.001)** | 43.033 **(< 0.001)** | 2.174 (< 0.25) |
| **nutrients x light/dark (1)** | 0.002 (> 0.25) | 0.107 (> 0.25) | 0.002 (> 0.25) | 0.262 (> 0.25) |
| **nutrients x time (1)** | 1.839 (> 0.1) | 0.544 (> 0.25) | 0.172 (> 0.25) | 2.332 (< 0.25) |
| **light/dark x time (1)** | 0.035 (> 0.25) | 2.300 (< 0.25) | 0.844 (> 0.25) | 0.502 (> 0.25) |
| **nutrient x light/dark x time (1)** | 0.158 (> 0.25) | 4.389 (< *0.1*) | 0.351 (> 0.25) | 1.608 (> 0.25) |

**Table 3.5.** Results of repeated measurement ANOVA on abundances of total heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophs (MNF) and bacteria with nutrient and light treatments as between-group factors and abundances on different sampling days (time) as within group factor, calculated with recalculated df values using 3 replicates instead of 4 for the calculation. This table is designed in the same way as Table 3.4. Results for MNF on day 1 and bacteria on day 3 should be considered with care since since a violation of variance homogeneity was detected for bacteria (day 3: Bartlett's $\chi^2$=31.042, p<0.001) and data were not normally distributed for MNF ((day 1): $\chi^2$=7.816, p=0.020).

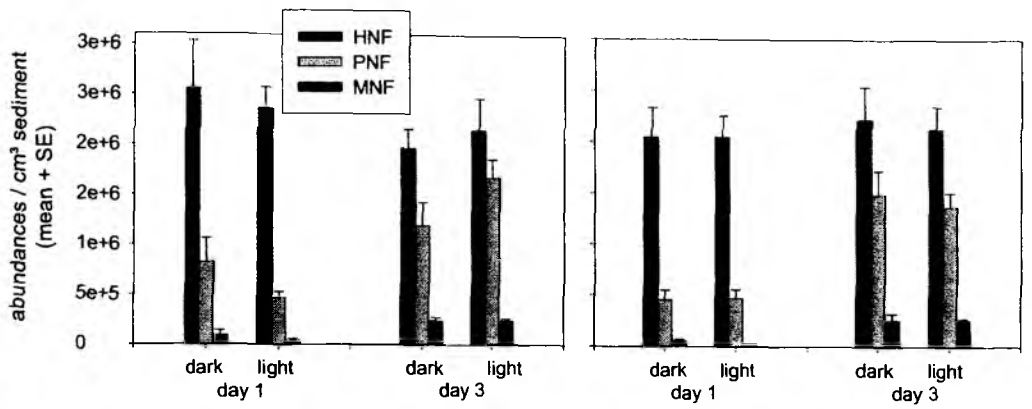



**Fig. 3.12.** Microbial community structure in nutrient depleted an in situ sediment in light and dark incubation on day 1 and day 3 of Experiment III. Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF) and mixotrophic nanoflagellates (MNF) per $cm^3$ sediment.

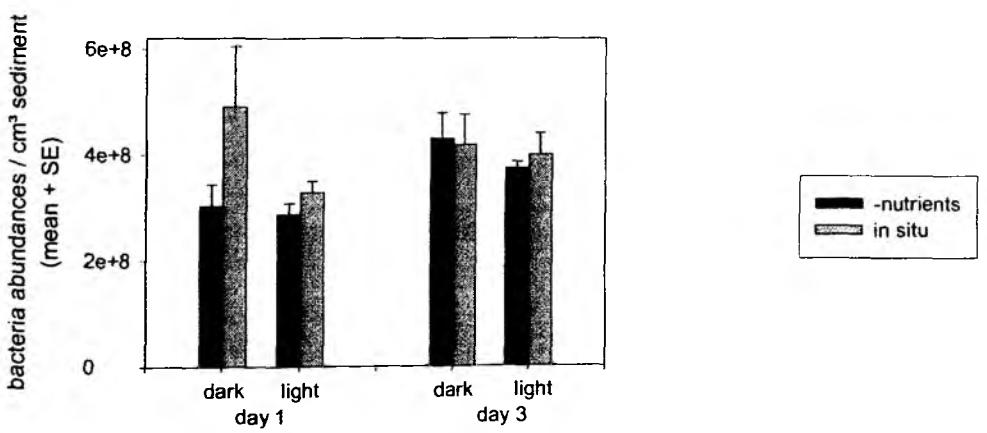



**Fig. 3.13.** Absolute abundances of bacteria per $cm^3$ sediment in light and dark incubations in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment III.

*Taxonomic composition*

Community composition changed in different treatments in the time course of the experiment (Fig. 3.16.). Chrysomonadida were presented with minor contributions compared to previous experiments, ranging from 2% to 8%. Only in the dark incubation on day 3 they contributed more than 20% to the total nanoflagellate community (Fig. 3.16.). In this treatment, the lowest taxonomic diversity was maintained; only 5 taxonomic groups were presented here in *in situ* and nutrient depleted sediment, whereas 10 taxonomic groups were found in light incubations on day 3 in *in situ* sediment and 8 groups in nutrient depleted

sediment. Again, differences in species composition mainly appeared between day 1 and day 3 and not between different treatments.

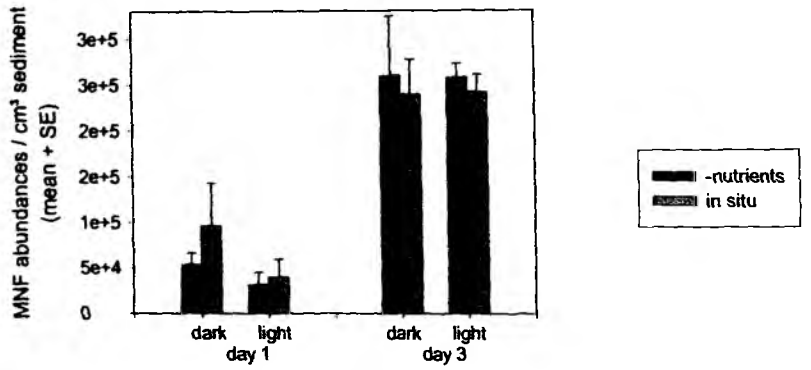



**Fig. 3.14.** Absolute abundances of mixotrophic nanoflagellates (MNF) per cm³ sediment in light and dark incubations in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment III.

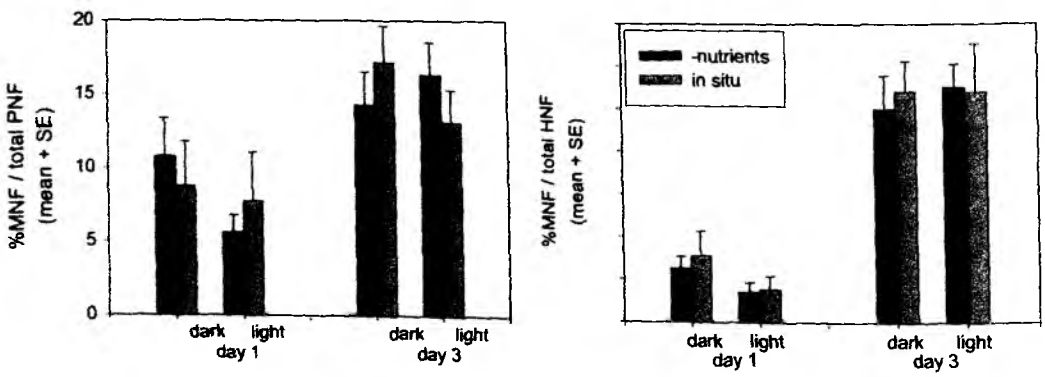



**Fig. 3.15.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates and the total heterotrophic nanoflagellates (HNF+MNF) in light and dark incubations in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment III.

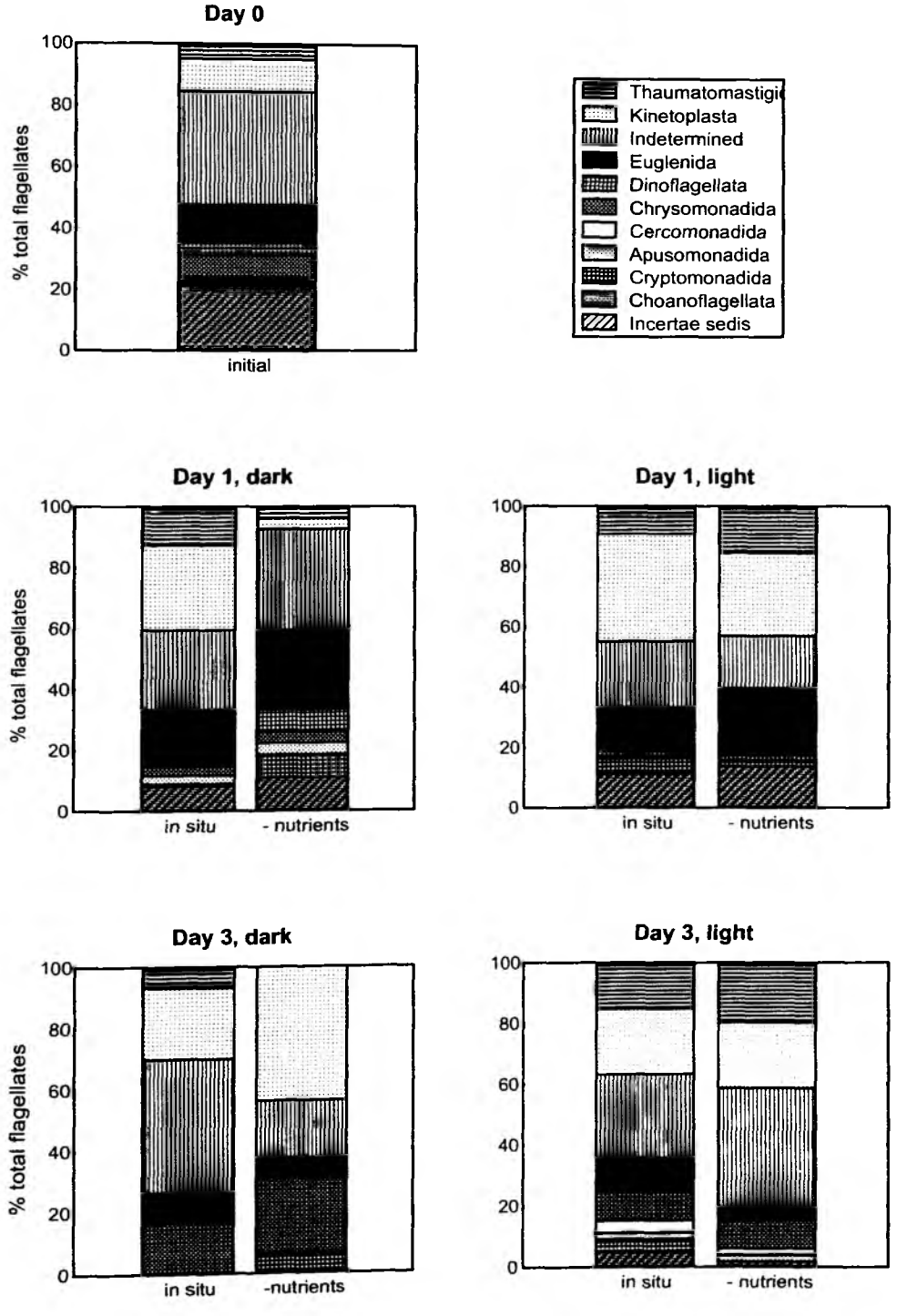



**Fig. 3.16**. Species composition in light and dark incubations in nutrient depleted and in situ sediment on day 1 and day 3 of Experiment III. Relative abundances of different taxonomic groups are calculated as % of the total flagellates.

## 3.4. Discussion

In all experiments, the nanoflagellate community was clearly dominated by HNF and detected mixotrophs contributed only minor portions to the flagellate community. Playing a more important role as primary producers than as bacterivores, their contributions to the total heterotrophic NF, phototrophic NF and total NF varied in different experiments and in different seasons. But overall, their quantitative importance was much lower than found in a variety of previous plankton studies (e.g. Berninger et al. 1992, Hall et al. 1993, Nygaard & Tobiesen 1993, Arenovski et al. 1995, Havskum & Riemann 1996, Jansson et al. 1997, Safi & Hall 1999, Sanders et al. 2000).

Regarding the manipulation of nutrients, MNF showed disparate responses in the experiments conducted in February (Experiment I and II), depending on light conditions and community composition. Phagotrophic activity has been reported for a variety of photosynthetic algal taxa and is particularly common in phototrophic chrysophytes, prymnesiophytes, and dinoflagellates (Boraas et al. 1988, Sanders & Porter 1988).

In the dark incubation (Experiment I), the initial mixotrophic community did not respond with increasing phagotrophic activity to nutrient limitation when photosynthesis was already light limited. However, within the 3 days of incubation, bacterivorous mixotrophs were probably favored in nutrient depleted sediments more than in *in situ* sediments, resulting in higher phagotrophic activities in nutrient depleted sediment in day 3. Chrysomonads increased drastically in nutrient depleted sediments from day 1 to day 3. Since chrysomonads are known to have a great number of mixotrophic species, it can be assumed that they were responsible for the increasing phagotrophic activity of the mixotrophic community. In the light incubation (Experiment II), the initial mixotrophic community responded to nutrient depletion. This effect leveled off after 7 days, possibly due to nutrient regeneration, and was not evident anymore after 24h incubation in the dark, where phagotrophic activity increased equally in nutrient depleted and *in situ* sediments. Obviously, light conditions generated phagotrophic activity in mixotrophs more than nutrient conditions and the effects did not add up, i.e. equal abundances of MNF with ingested FLB were found in both nutrient depleted and *in situ* sediments. Chrysomonads, increased considerably from day 1 to day 7 and 8, but light favored them equally irrespective of nutrient conditions, which might have been due to nutrient regeneration within the 7 days incubation. Assuming chrysomonads to provide the major part of the mixotrophic community, equally increasing contributions to the flagellate community in both nutrient treatments would explain equal phagotrophic activities when incubated in the dark. The fact that initial MNF communities on day 1 responded to nutrient

limitation on day 1 in Experiment II, but not in Experiment I is most likely due to different light conditions. In Experiment II, photosynthesis was only nutrient limited and part of the MNF was able to respond. In Experiment I, photosynthesis was both light and nutrient limited and phagotrophic activity was generated more by light limitation than by nutrient limitation. In order to properly distinguish between both factors, it was necessary to conduct Experiment III, where both factors were manipulated in a factorial design.

Surprisingly, neither light nor nutrient conditions affected phagotrophic activities of MNF. In this experiment, chrysophytes did not play the same quantitative role as in Experiments I and II and the flagellate community was much more diverse with regard to taxonomic groups in October (Exp. III) than in February (Exp. I and II). Most likely the mixotrophic community in October consisted of a different species pool compared to February (Exp. I and II). Light conditions at Falckenstein Beach in the beginning of October are much better than light conditions in February, when the period of day light is much shorter. In the winter season, it could be therefore more important for mixotrophs to switch from photosynthesis to phagotrophy at dark than in the beginning of October, where the day light period is much longer. However, overall contributions of MNF to the flagellate community were slightly higher in October than in February, indicating that light conditions did not influence total mixotrophic contributions.

Environmental factors as well as predator – prey dynamics influence benthic microbial community structure, resulting in oscillations on small temporal and spatial scales and in seasonal dynamics (Epstein 1997b, Findlay & Watling 1998). Therefore it is not surprising to find different microbial community structures in February and October and also different dynamics of mixotrophs, which can not be easily explained or reduced to particular environmental factors. My findings are consistent with a variety of plankton studies, investigating particular mixotrophic species or mixotrophic contributions in natural communities.

Studies on mixotrophic species revealed a high variety of nutritional modes with different relative contributions of phagotrophy and photosynthesis, being species specific and dependent on several environmental factors. For instance, different feeding responses to light intensity and inorganic nutrients have been observed in mixotrophic dinoflagellates. Feeding by *Fragilidium subglobosum* was stimulated by light under dim light conditions, but was inhibited under high light intensities (Skovgaard 1996, Hansen & Nielsen 1997). Feeding in *Prorocentrum minimum*, however, was stimulated by light, but inhibited by addition of inorganic nutrients (Stoecker et al. 1997). Also in other taxonomic groups a high variability in

feeding strategies were found, like the mixotrophic chrysophytes *Dinobryon cylindricum* and *Poterioochromonas malhamensis*. The first one was found to be an obligate phototroph, requiring light in addition to bacterial prey (Caron et al. 1993), whereas the latte is predominantly heterotrophic, showed increasing ingestion rates with decreasing light regimes (Holen 1999). Jones et al. (1993) demonstrated increasing ingestion rates inversely proportional to irradiance in *Chrysochromulina brevifilum* (Prymnesiophyceae). Phosphate limitation has also been shown to increase feeding in *C. brevifilum* and other mixotrophic *Chrysochromulina* species *C. ericina* and *C. polylepis* (Jones et al. 1993, Nygaard and Tobiesen 1993). These examples show the variety of different functional types of mixotrophy and indicate that information on the physiological ecology of mixotrophic protists is crucial to understand their ecological role in food webs and their impact on trophodynamics and food web structure (Jones 1994, Stoecker 1991, Stoecker & Michaels 1991, Turner & Roff 1993, Reimann et al. 1995, Jones 1997). Different functional types of mixotrophy should affect the total productivity of the microbial food web in different ways (Stoecker 1998) as well as the trophic transfer from the microbial food web to metazoa (Holen & Boraas 1995), or competition among phytoplankton (Rothhaupt 1996a, Thingstad et al. 1996, Stoecker et al. 1997). Apart from different types of mixotrophy, also the relative balance between different carbon and energy sources, such as DOC, bacterial prey and light availability are important for food web implications in the system (Stoecker 1998, Jones 2000). Due to the variety of nutritional characteristics of mixotrophic flagellates and the different factors influencing their role in microbial dynamics and the energy flow in a system, it is not possible to regard mixotrophy simply as nutritional strategy in oligotrophic environments or in deeper waters, where photosynthesis is nutrient or light limited, respectively. For instance, Hall et al. (1993) measured high rates of bacterivory by phytoflagellates in coastal waters during an upwelling event when nitrogen and phosphorus concentrations were high. Sanders et al. (2000) found more mixotrophic nanoflagellates at a coastal ocean site of Georges Bank (off the northeast USA), where tidal mixing maintains high concentrations of dissolved inorganic nutrients, compared to the oligotrophic Sargasso Sea, again showing that the acquisition of major nutrients is only one of several possible inducements of phagotrophic behavior among algal species (Boraas et al. 1988, Caron et al. 1990, Sanders et al. 1990, Sanders 1991b, Caron et al. 1993, Rothhaupt 1996a). Bird & Kalff (1986) found mixotrophic flagellates to be abundant in metalimnic phytoplankton maxima and attributed their high abundances to low light intensities limiting photosynthesis. On the other hand, Holmgren (1983) found pigmented flagellates to dominate in shallow clear-water lakes, showing that mixotrophic flagellates are

not restricted to low-light environments. In fact, most field studies and grazing experiments have failed to show an increasing clearance rate at low light intensities (Bird & Kalff 1987, Arenovski et al. 1995).

Uncertainty in the factors inducing phagotrophy in different algal species makes it difficult to predict where and when mixotrophy will be an important ecosystem process (Sanders et al. 2000). In addition, different factors influence mixotrophy on different levels of organization. Environmental factors affect the relative importance of photosynthesis and phagotrophy in individual species, but they may as well favor particular populations of mixotrophs, which influence dynamics in the whole microbial community. These factors also influence the community itself, which can in turn affect mixotrophic populations within the community. An array of environmental factors acts differently on all three levels (individual, population, community) on different temporal scales, making it almost impossible to make predictions about the importance of mixotrophs in natural systems. These aspects are further discussed in Chapter 6.

Results of the present study demonstrated that also benthic mixotrophs are able to respond to changing environmental conditions with a switch of their nutritional mode and to contribute different portions to primary production and bacterivory, although in a lower quantitative range compared to previous plankton studies. In the experiments described so far, I investigated only one type of sediment at Falckenstein Beach in the surface layer of 3mm. Sediment characteristics are known to influence microbial community structure (e.g. Alongi 1986, 1990, Fenchel 1996, Watermann 1999). Since light conditions change with sediment depth as well as other physical and chemical gradients, mixotrophy might play a different role in deeper sediment layers. Therefore, mixotrophic occurrence and significance were investigated in different sediment types and depths at Falckenstein Beach, described in Chapter 4.

# Chapter 4

# Variations in the occurrence and ecological significance of mixotrophic nanoflagellates in different sediment types and depths at Falckenstein Beach, Western Baltic Sea

## 4.1. Introduction

Benthic environments such as sediments are characterized by steep vertical and sometimes horizontal gradients of light, oxygen, nutrient concentrations and other physical and chemical factors (Jørgensen & Revsbech 1985, Revsbech & Jørgensen 1986, Kühl et al. 1994). Those gradients generate a pronounced heterogeneity and influence the distribution of microorganisms due to the requirement of different physiological adaptations for the occupation of special ecological niches. Vertical gradients in sediments result from a variety of biotic and abiotic factors. Physical properties of sediments are potentially important in determining the community structure of benthic protists. Fenchel (1996) postulated grain size and interstitial space to influence the occurrence and abundance of different ciliate species. Grain size was also found to affect competition between diatoms and cyanobacteria (Watermann et al. 1999). Alongi (1986) found flagellates to be the most dominant group of protozoa in sediments that were sufficiently fine to exclude larger ciliates. Furthermore, it was assumed that the amount of interstitial water is the most critical factor controlling benthic microfaunal densities in tropical sediments (Alongi 1990), where sediments with a higher compaction can support fewer protozoans in the interstices. In contrast Gasol (1993) reported the highest densities of heterotrophic nanoflagellates in lake sediments with low water contents.

Light propagation in sediments, which is also dependent on sediment structure and grain size (Kühl et al. 1994), is a key parameter for microbenthic photosynthesis. Photosynthesis of microflora in the euphotic zone of marine sediments may contribute significantly to the total primary production in shallow areas (Revsbech & Jørgensen 1983). Oxygen is not only released into the sediment but also into the overlying water (Revsbech et al. 1980, Yallop et al. 1994, Reay et al. 1995). This is in contrast to other sea beds, which are situated below the photic zone, where the supply of oxygen originates solely from the water column. In comparison to pelagic systems, light in microbenthic environments is subject to intense

absorption and multiple scattering due to the much higher density of sediment particles and microalgae (Kühl & Jørgensen 1994). This high optical density results in a euphotic zone that ranges from a few tenths of a millimeter to a depth of several mm (Jørgensen et al. 1983). A number of studies have demonstrated protistan community structure to change with sediment depth (Hondeveld et al. 1994, Berninger & Epstein 1995, Starink et al. 1996b). It can be assumed that light propagation in sediments, which is a crucial factor for phytoflagellates, will also influence mixotrophic community structure and the relative importance of photosynthesis and phagotrophy in particular mixotrophic organisms.

In previous experiments (see Chapter 2 and 3), the sediment surface layer of 3mm was sampled, approximately 2m behind the shoreline at Falckenstein Beach in the Western Baltic Sea. The sediment at this site is very coarse, but becomes finer with increasing water depth and distance from the shoreline. Sediment structure was shown to influence vertical physical and chemical gradients as well as microbial community structure (see above). Therefore, it can be assumed that different sediments at Falckenstein Beach differ in their vertical gradients and thereby also in their community composition. Mixotrophic flagellates occur in a variety of different taxonomic groups (e.g. Sanders & Porter 1988), and their nutritional mode, i.e. relative contributions to photosynthesis and phagotrophy, is species specific and dependent on environmental factors such as light level and nutrient concentrations (Nygaard & Tobiesen 1993, Keller et al. 1994, Urabe et al. 1999, Urabe et al. 2000). As microbial community structure is likely to change along small scale horizontal and vertical gradients in the same benthic system at Falckenstein Beach, the quantitative importance and ecological role of mixotrophic flagellates might comparably change.

In the present study three different sediment types, exhibiting different sediment structures were investigated with a vertical resolution of three different layers within the first cm of surface sediment. Therefore, this study provides records of variation in microbial community structure with emphasis on the quantitative role of mixotrophic nanoflagellates along a horizontal and a vertical gradient in sediment of Falckenstein Beach in the Western Baltic Sea.

## 4.2. Material and Methods

### 4.2.1. Experimental design and sampling procedure

The FLB grazing experiment was conducted in July 2003 with sediment cores from Falckenstein Beach, Kiel Fjord, Western Baltic Sea (10° 11' 40''E, 54° 24' 23''N). Since all previous experiments with sediment from Falckenstein Beach were conducted in early spring, autumn or winter, where minor MNF contributions were found, a point of time within the summer vegetation period was chosen to investigate whether phytoflagellates and mixotrophs play a greater role at this time of the year. The experiment was designed to investigate maximum abundances of potentially mixotrophic nanoflagellates in a vertical gradient, resolved in 3 different sediment layers (0-3mm, 3-6mm and 6-9mm depth) in 3 different sediment types along a horizontal gradient of Falckenstein Beach. Since more mixotrophs with ingested FLB could be identified in dark incubations in prior experiments, the FLB grazing experiment was conducted in the dark. The different sampling sites were located 2m, 5m and 10m behind the shoreline, representing the mean high water level, and are called Station 1 (S1, 2m), Station 2 (S2, 5m) and Station 3 (S3, 10m) in the following course of the chapter.

In order to collect 4 replicate sediment cores per station, the beach was divided into 20 patches and 4 patches (for 4 replicates) were randomly chosen for sampling to account for the heterogeneity of the sediment along the beach. This was done for every station, respectively. Within each patch, 6 sediment cores were collected; two for the FLB grazing experiment (t0 and t16, see below), one additional core to determine initial bacterial abundances for the calculation of FLB numbers to be inoculated, and 3 cores to determine the proportion of organic matter for every depth. Furthermore, sediment samples were taken at each station for life counts (see Chapter 3), oxygen profiles (see below) and sieving analyses in order to characterize the physical characteristics of sediment from different locations and small scale spatial variations within locations.

Sediment cores were transferred to the laboratory, where further processing took place. Since sampling had to be destructive, 2 cores had to be taken for t0 and t16 sampling in the FLB experiment, respectively. The FLB grazing experiment was conducted in the same way as described in Chapter 3 and in Chapter 2 for Experiment I and II. Sediment cores were extruded in the laboratory and 3mm thick sediment slices of different layers (0-3mm, 3-6mm and 6-9mm) were transferred into tissue culture plates (see Chapter 2) with 2ml of sterile filtered seawater (SSW). From one additional core, bacterial abundances were calculated for

each station, and FLB concentrations approximating 25% of the natural bacterial abundances, respectively, were added to the slurries. After FLB addition, t0 samples were fixed with ice cold glutardialdehyde at a final concentration of 1.5%. The second set of samples incubated with FLB was completely covered with aluminum foil to account for complete darkness and incubated in a climate chamber for 16h at 18°C. After 16h, t16 samples were fixed and all samples stored at 4°C in the dark until further processing.

### 4.2.2. FLB preparation

As in the previous experiments, sediment from the sampling site at Falckenstein Beach was collected one week before conducting the experiment. Natural bacteria were isolated from this sediment and monodispersed natural benthic FLB were prepared as described in Chapter 2. Only one type of FLB was prepared for all three stations, with bacteria being isolated and mixed from every sediment type.

### 4.2.3. Sample processing

Samples were processed in the same way as described in Chapter 2 and 3. The sediment was transferred out of the cell wells into a 300ml polystyrene cell culture flask, diluted subsamples collected on 0.2µm black polycarbonate filters and stained with DAPI for 5min at a final concentration of 5µg/ml. Filters were mounted on slides, sealed with paraffin wax and stored at -20°C until microscopic analysis.

### 4.2.4. Sediment analysis

At each station, sediment was collected from the first cm of the sediment surface layer for sieving analysis, thus pooling the sediment from the different layers investigated. It was not possible to resolve the different sediment layers for sieving analysis, since a sediment volume of 150 $cm^3$ is needed for a representative analysis (Batel 1964), which was not possible to take with the available number of sediment cores. Sediment structure did not visibly change within the first cm of surface sediment (pers. observation). Sediment samples were dried at 70°C overnight until their weight was constant. Sediment from every station was weighed, sieved through 6 sieves with different mesh sizes (0.063mm, 0.5mm, 1mm, 2mm and 3.5mm) and size fractions weighed to calculate weight proportions of different grain sizes. For determination of organic matter, the 3 additional replicate sediment cores (see above) from each station were extruded in the laboratory and sediment slices from each depth (0-3mm, 3-6mm, 6-9mm) transferred into pre-nealed aluminum plates with a diameter of 6cm. Sediment

was weighed and then dried within the plates at 70°C for 5h. After weighing the dried sediment again, it was transferred into an oven and incinerated for 16h at 550°C. Samples were cooled in an excicator, weighed again and incinerated particulate organic matter calculated.

For oxygen profiles, sediment cubes (12.5cm x 8.5cm x 10cm) were cut out of the sediment and transferred into acrylic glass chambers, so that the vertical structure of the sediment cube was maintained. These chambers were transferred to the laboratory and oxygen profiles were measured at a vertical resolution of 100µm in a climate chamber at 18°C and a light intensity of $60\mu Em^{-2}s^{-1}$, employing Clarke-type oxygen microelectrodes, according to the description in Revsbach & Jørgensen (1986).

### 4.2.5. Counting

Flagellates, diatoms, cyanobacteria, bacteria and FLB were counted on the filter preparations with an epifluorescence microscope (Leica/Leitz DMRB) at 1000x magnification in the same way as in previous experiments described in Chapter 2 and 3, using a blue filter set for counting nanoflagellates and diatoms (Leica/Leitz filter set 13), a green filter set for counting cyanobacteria (Leica/Leitz filter set N) and a UV filter set for counting bacteria (Leica/Leitz filter set A). Furthermore, diluted sediment subsamples (1:10) were analyzed in order to classify flagellates into major systematic groups, using a live counting technique (e.g. Gasol 1993, Dietrich & Arndt 2000, see Chapter 3). Three aliquots of 5µl to 20µl were counted per station and depth on a slide under an upright phase contrast microscope at 400x magnification (Leitz Dialux 20, Wetzlar Germany).

### 4.2.6. Statistical Analysis

In order to test for differences in components of the microbial community at different stations and depths, a two-factor ANOVA (station x depth) was conducted on abundances of HNF, PNF, MNF, diatoms, cyanobacteria and bacteria, respectively. Normal distribution and homogeneity of variances were tested with a Chi-Square-Test and with a Bartlett-Chi-Square-Test, respectively. Posthoc tests to determine at which particular stations and depths species abundances significantly differed were conducted with Tukey's HSD test.

## 4.3. Results

### 4.3.1. Sediment analysis

The stations sampled at Falckenstein Beach clearly differed in sediment structure/grain size, getting increasingly finer with increasing distance from the shore line (Fig. 4.1.). Size fraction 0.5-1mm increased from station 1 to 3 from 35% to more than 80%, while bigger size fractions decreased. Portions of particulate organic matter were low and ranged from 0.5% to 1.2% per sediment layer at each station, with slightly higher portions at S1 compared to S2 and S3 (Fig. 4.2.). At S1, the sediment was 100% oxygen saturated until a depth of 3mm, after which oxygen saturation declined, but was still evident at 10mm depth with an oxygen saturation of 7%. At S2, oxygen saturation declined to depth of 5mm and at S3 to 4.5mm, below which the sediment was oxygen depleted. At S2, oxygen saturation immediately declined after a surface maximum of more than 100%, whereas at station 3, consisting of the finest sediment, the sediment was completely saturated with oxygen in the first 2mm, after which a steep decline led to oxygen depletion at about 4mm (Fig. 4.3.).

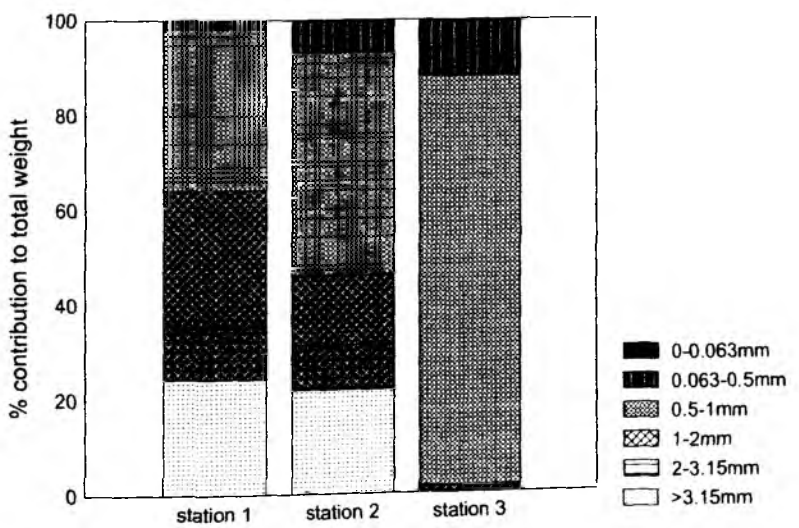



**Fig. 4.1.** Weight proportions of different size fractions of sediment grains at Station 1, 2 and 3.

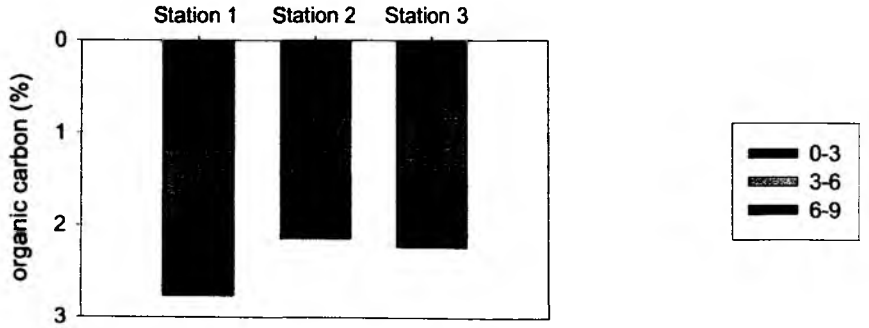



**Fig. 4.2.** Weight proportion of organic carbon, contributed to the total sediment at Station 1, 2 and 3 in all sediment layers (0-3mm, 3-6mm, 6-9mm depth) investigated.

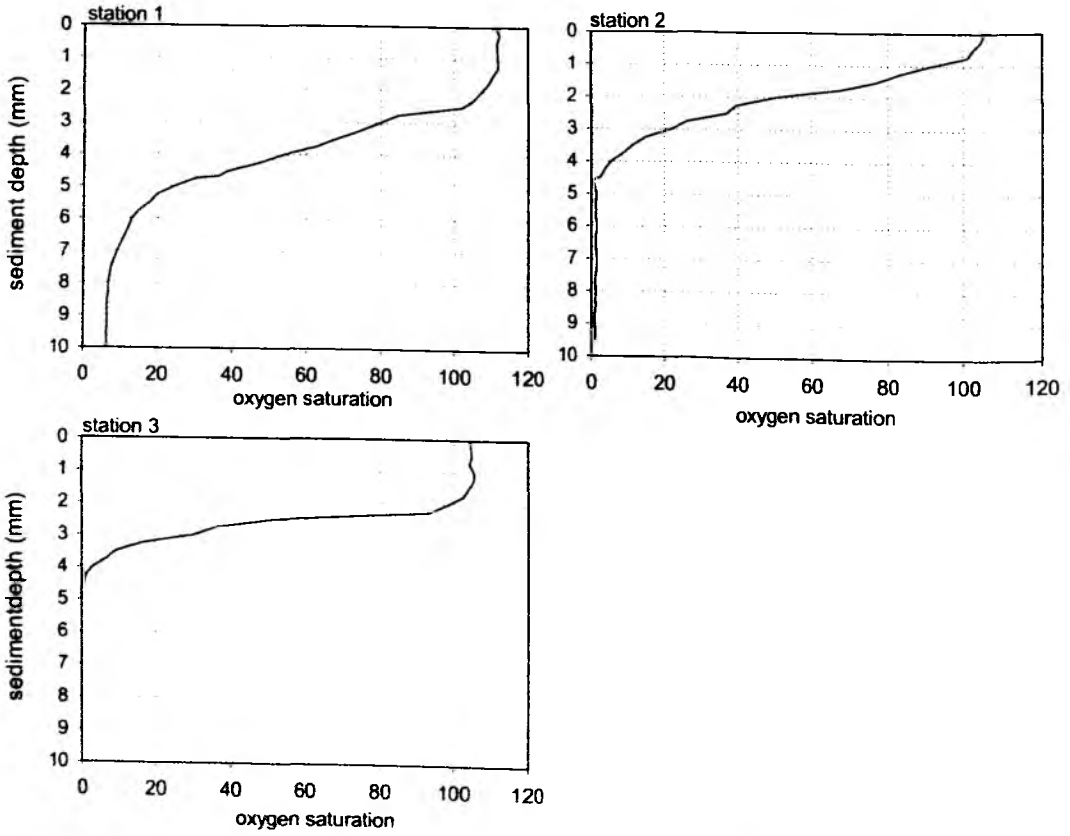



**Fig. 4.3.** Oxygen profiles measured with a spatial resolution of 0.1mm in the first 10mm of sediment of Station 1, 2 and 3.

### 4.3.2. Abundances of organisms and species composition

*Abundances*

All nanoflagellate communities at all stations and sediment depths were clearly dominated by HNF, which contributed around 80% to the nanoflagellate community (Fig. 4.4.). PNF contributed about 20% to the nanoflagellates, and MNF only minor portions of 1-2% (Fig. 4.4., Table 4.2.). Abundances of all organisms significantly increased from S1 to S3, (Fig. 4.5. Table 4.1.) being significantly higher at S3 than at S1 at all sediment depths (Tukey's HSD, $p<0.05$), except for bacterial abundances. Abundances of HNF, PNF, diatoms and cyanobacteria ranged from $1x10^5$ at S1 to $2x10^6$ at S3, MNF abundances from $1x10^4$ to $7x10^4$ and bacterial abundances from $1x10^8$ to $4x10^8$ (Fig. 4.5.). HNF abundances in the sediment layers 0-3mm and 6-9mm at S3 were significantly higher than in all sediment layers of S2 (Tukey's HSD, $p<0.001$). Abundances hardly varied with depth; at S1, HNF abundances slightly increased from the surface to the intermediate sediment layer, whereas at S3 abundances first declined and increased again in the deepest layer (Fig. 4.5.). PNF abundances were significantly higher at S3 than at S2 for all sediment depths (Tukey's HSD, $p<0.001$). In addition to differences between stations, sediment depth also influenced PNF abundances slightly insignificant at a level of $p=0.082$, with abundances at S3 in the surface layer (0-3mm) being significantly higher than in the second layer (3-6mm) at a p-level of $p=0.052$. Also at S2, there was a trend of decreasing abundances with depth, whereas at S1, PNF abundances increased in the intermediate sediment layer and decreased again in the deepest layer (Fig. 4.5.). MNF abundances in the surface layer (0-3mm) of S3 exceeded abundances in all layers of S2 (Tukey's HSD, $p<0.02$). Within each station, MNF abundances hardly changed with depth at S1, but increased from the surface to the intermediate sediment layer at S2, to decrease again in the deepest layer. At S3, MNF abundances decreased with depth. Diatom abundances in the surface and the deepest layer of S3 exceeded abundances of all layers at S2 (Tukey's HSD, $p<0.04$). Abundances hardly varied with depth (Fig. 4.5.). Cyanobacteria in the surface layer (0-3mm) of S3 significantly exceeded abundances in the intermediate (3-6mm) and the deepest (6-9mm) layer of S2 (Tukey's HSD, $p\leq0.02$). At S2 and S3, abundances decreased with depth. Bacterial abundances did not increase to the same extent from S1 to S3 as described for other microbes before. However, abundances in the deepest layer of S3 significantly exceeded abundances in the surface and the deepest layer of S1 (Tukey's HSD, $p\leq0.003$) and abundances in all sediment layers of S3 and the deeper layers of S2 (3-6mm, 6-9mm) exceeded abundances in the intermediate layer of S1 at a level of $p\leq0.073$ (Tukey's HSD) (Fig. 4.5.).

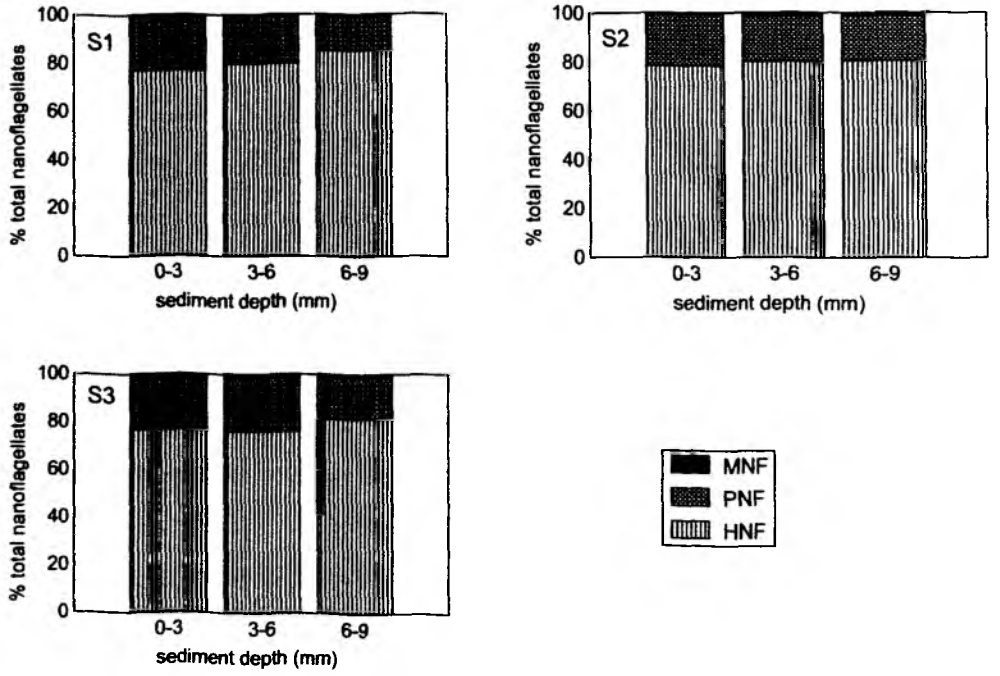



**Fig. 4.4.** Relative abundances of heterotrophic (HNF), phototrophic (PNF) and mixotrophic (MNF) nanoflagellates as percentage of the total nanoflagellates at Station 1, 2 and 3 in all sediment layers (0-3mm, 3-6mm, 6-9mm depth) investigated.

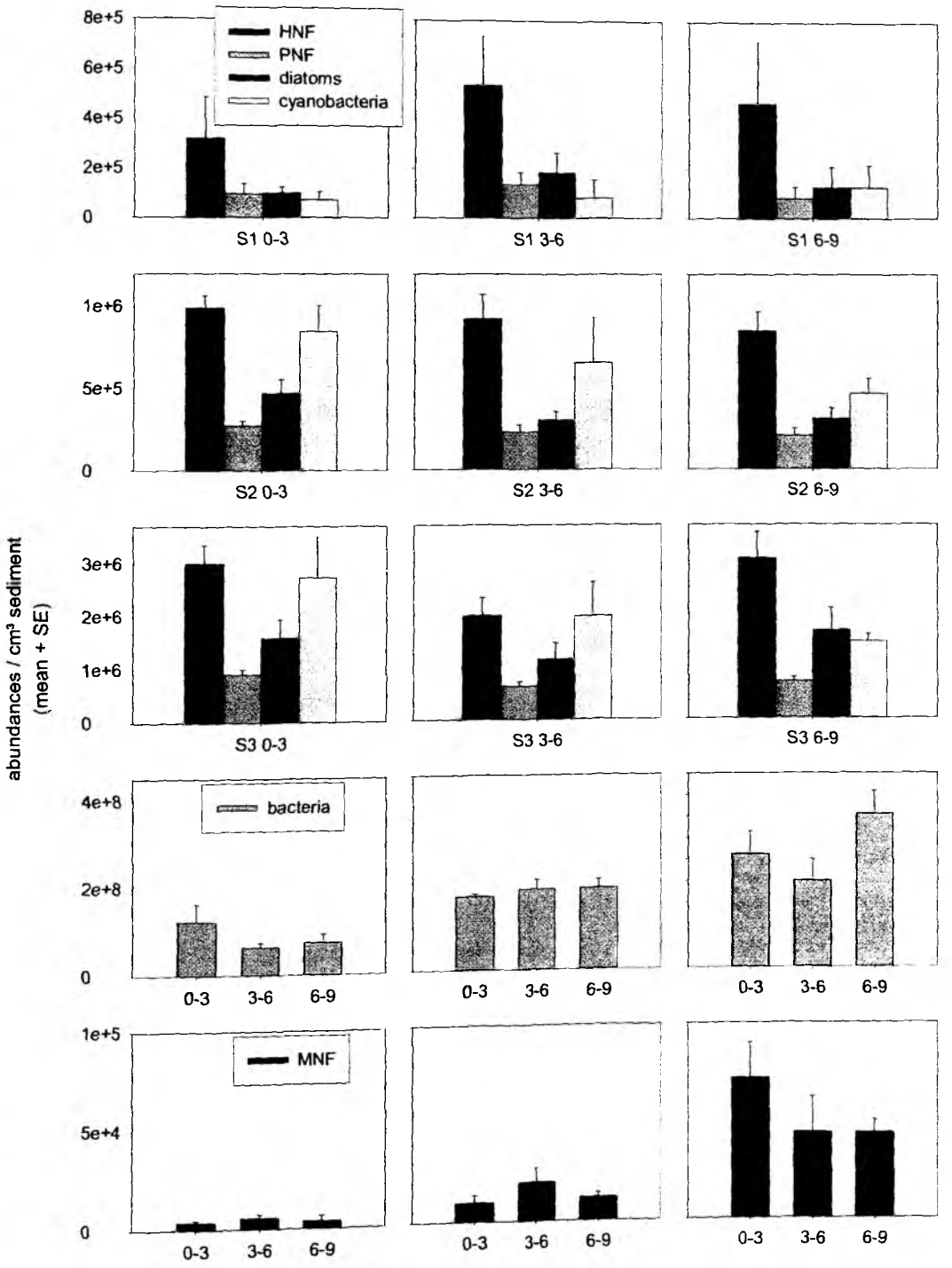



**Fig. 4.5.** Microbial community structure at Station 1, 2 and 3 in all sediment layers (0-3mm, 3-6mm, 6-9mm depth) investigated. Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophic nanoflagellates (MNF), diatoms and bacteria per $cm^3$ sediment in dark incubations with natural $FLB_{nat}$. In Experiment IV, abundances after 16h of incubation time are presented.

| Factor | total HNF | PNF | MNF | diatoms | cyanobac. | bacteria |
|---|---|---|---|---|---|---|
| df | 27 | 27 | 27 | 27 | 27 | 27 |
| station (2) | 57.057 **(< 0.001)** | 95.981 **(< 0.001)** | 34.407 **(< 0.001)** | 44.759 **(< 0.001)** | 36.875 **(< 0.001)** | 24.612 **(< 0.001)** |
| depth (2) | 1.255 (0.301) | 2.750 *(0.082)* | 0.217 (0.807) | 0.393 (0.679) | 1.286 (0.293) | 1.819 (0.182) |
| station x depth (4) | 2.026 (0.119) | 1.959 (0.130) | 1.495 (0.231) | 0.789 (0.543) | 0.482 (0.749) | 1.726 (0.173) |

**Table 4.1.** Results of a 2-factor ANOVA on abundances of total heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophic nanoflagllates (MNF), diatoms, cyanobacteria and bacteria, with station (1-3) and sediment depth (0-3mm, 3-6mm, 6-9mm) as independent factors. The table gives the F-ratios (with significance levels in parentheses) for the main factors. The degrees of freedom for the effect terms are given in parentheses for each effect, for the error term in the row on top of the analysis. Effects significant at $p<0.05$ are printed in bold, trends with $p<0.1$ are printed in italics. The results for MNF and diatoms should be considered with care since a violation of variance homogeneity was detected for MNF (Bartlett's $\chi^2=17.726$, $p=0.023$) and data were not normally distributed for diatoms ($\chi^2=14.201$, $p=0.048$).

| Station | %MNF S1 | %MNF S2 | %MNF S3 | %HNF S1 | %HNF S2 | %HNF S3 |
|---|---|---|---|---|---|---|
| 0-3mm | 1.4 ± 0.3 | 0.8 ± 0.3 | 1.8 ± 0.5 | 11.64 ± 1.0 | 7.2 ± 1.1 | 9.4 ± 0.8 |
| 3-6mm | 1.0 ± 0.2 | 1.7 ± 0.5 | 1.6 ± 0.5 | 7.6 ± 0.9 | 8.0 ± 0.6 | 6.6 ± 1.0 |
| 6-9mm | 0.50 ± 0.3 | 1.2 ± 0.3 | 1.2 ± 0.2 | 5.2 ± 0.9 | 6.9 ± 0.9 | 6.8 ± 1.1 |

**Table 4.2.** Relative abundances of mixotrophic nanoflagellates as % of the total nanoflagellates (%MNF) and heterotrophic nanoflagellates with ingested FLB as % of the total heterotrophic nanoflagellates (%HNF + FLB / total HNF) at different stations (S1 - S3) and sediment depths (0-3mm, 3-6mm, 6-9mm). Values present mean (n=4) ± standard error.

*Contributions*

Although absolute abundances of MNF differed at S1-3, relative abundances hardly changed. MNF contributed maximum portions of 2% to the total NF (Table 4.2.). At S1 and S3, MNF contributed 5% and 8% to the total PNF, respectively, with equal portions at all sediment depths (Fig. 4.6.). At S2, MNF contributions to the total PNF increased from the surface (0-3mm) to the intermediate (3-6mm) sediment layer from 5 to almost 10%, slightly decreasing in the deepest layer. Contributions to the total grazers ranged from 1% to 3% at all stations in all layers (Fig. 4.6.). Grazing activity of HNF (HNF+FLB/total HNF) ranged from 5% to 12%, being highest in the surface layer of S1, and decreasing with sediment depth at S1 and S3. At S2, grazing activity hardly changed with depth (Table 4.2.).

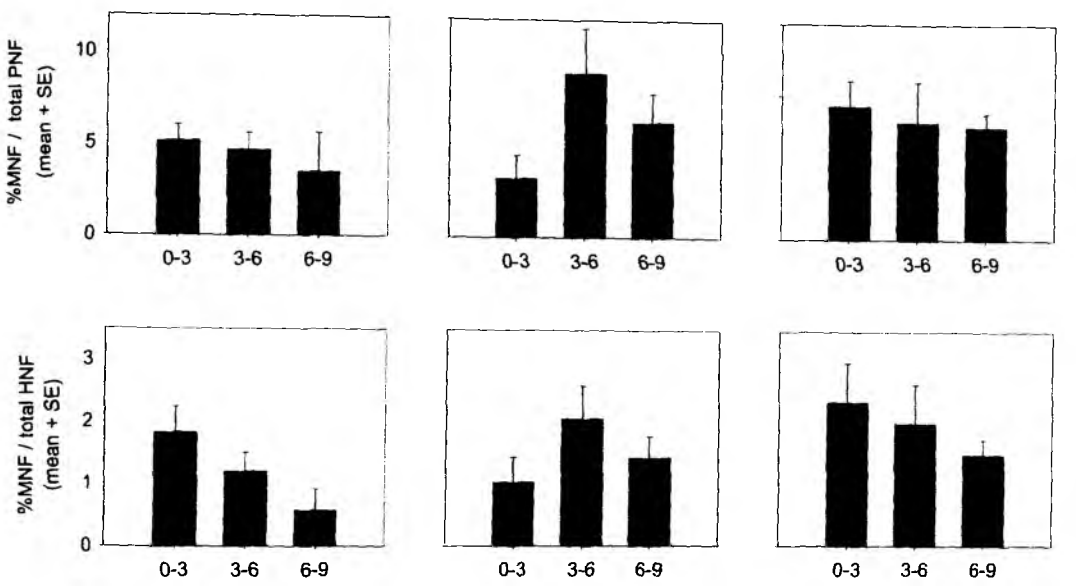



**Fig. 4.6.** Relative abundances of mixotrophic nanoflagellates as percentage of the total phototrophic nanoflagellates (PNF) and the total heterotrophic nanoflagellates (HNF) at Station 1, 2 and 3 in all sediment layers (0-3mm, 3-6mm, 6-9mm depth) investigated.

*Taxonomic composition*

The species composition changed with increasing depth at all stations (Fig. 4.7.). At S1, contributions of Thaumatomastigida, Euglenida and Chrysomonadida increased with depth, whereas Cryptomonadida, and Kinetoplasta decreased. Dinoflagellates were only present in the intermediate sediment layer (3-6mm). Diversity, which here is defined as number of taxonomic groups hardly changed with sediment depth. Also at S2, diversity did not change with sediment depth, only contributions of different taxonomic groups. Euglenida were not present in the surface layer, but were recorded in increasing abundances in the intermediate and the deepest layer, whereas abundances of mostly phototrophic Chrysomonadida decreased in the deepest layer. Cryptomonadida slightly increased in deeper sediment layers (Fig. 4.7). In the surface and the intermediate sediment layer, S3 exhibited the highest diversity of all stations with 9 different taxonomic groups. Diversity decreased in the deepest layer, and only 5 taxonomic groups were still present (Fig. 4.7.). Abundances of Thaumatomastigida decreased with depth, whereas Kinetoplasta increased in equal measure. Euglenida slightly increased from the surface to the intermediate layer, but disappeared in the deepest layer, whereas Cryptomonadida and Incertae sedis slightly decreased from the surface to the intermediate layer, but also disappeared in the deepest layer.

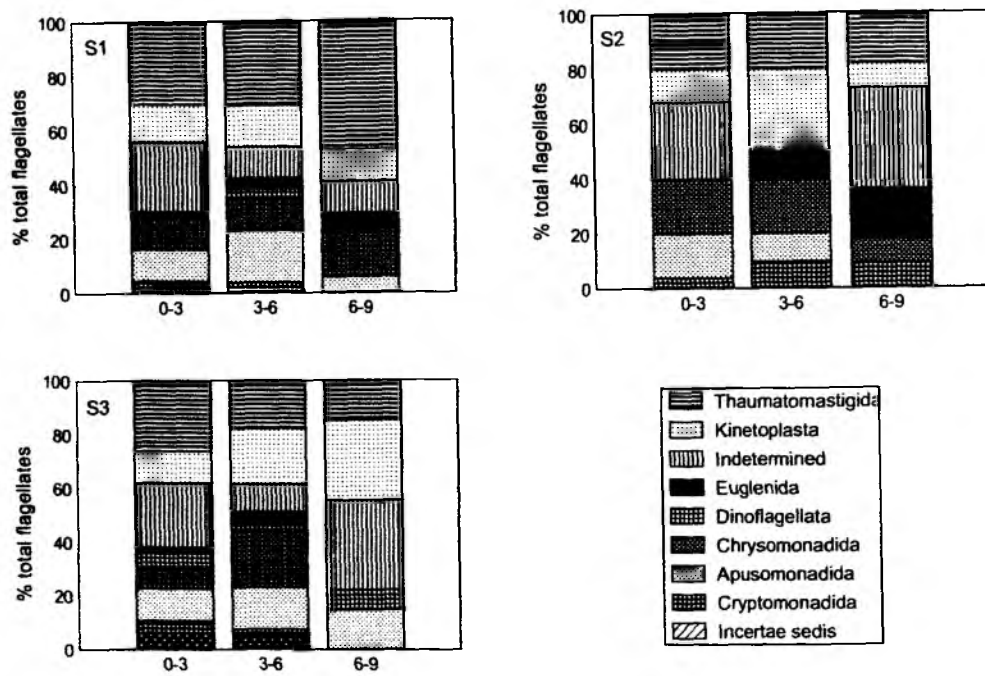



**Fig. 4.7.** Species composition at Station 1, 2 and 3 in all sediment layers (0-3mm, 3-6mm, 6-9mm depth) investigated. Relative abundances of different taxonomic groups are calculated as % of the total flagellates.

## 4.4. Discussion

The sediments sampled at Falckenstein Beach clearly differed with respect to sediment structure/grain size and light propagation accompanied by oxygen saturation along the vertical gradient. As expected, microbial community compositions changed with increasing sediment depth, which was most pronounced at S3. Diversity of taxonomic groups decreased with decreasing oxygen saturation and heterotrophic groups like Kinetoplasta increased, whereas mostly phototrophic groups like Chrysomonadida disappeared. Changes between different stations were also evident, but were more pronounced along vertical gradients. These changes in the entire microbial community structure were probably accompanied by changes in the mixotrophic community structure (see below). Absolute abundances of all species increased except for bacteria from S1 to S3. It can be assumed that organisms at S1 are subject to continuous water movement directly behind the shoreline, and are resuspended in the water column or migrate into deeper sediment layers. The water becomes much calmer with increasing depth and distance from the shore. Also the sediment grain size probably influenced species abundances and my results correspond to the findings of Gasol et al.

(1993), who found the highest densities of heterotrophic nanoflagellates in fine lake sediments with low water contents.

The sediment was increasingly finer with increasing distance from the shore line, which affected light propagation along the vertical gradient and thus photosynthetic oxygen production. Oxygen concentration at and just underneath the sediment surface was higher than in the overlying water (exceeding 100% oxygen saturation, 100% being calibrated just above the sediment surface in the water column). Therefore, it can be assumed that oxygen, measured in the sediment, was produced by photosynthetic activity and had not diffused from the water column into the sediment (Berninger & Huettel 1997). Light intensities are closely related to photosynthetic oxygen production in sediments, and mechanisms of light propagation were also reflected in the oxygen profiles of the sediments investigated. Light intensity is a crucial factor in determining mixotrophic abundances and feeding behavior. In contrast to most oceanic and clear coastal waters, where irradiance reflectance is only a few %, reflectance is high in sediments, and the light field becomes more diffuse due to the high density of scattering material (Kühl & Jørgensen 1994). Sediment microalgae therefore live in a highly diffuse light field and receive light from all directions around the cells (Jørgensen & Des Marais 1988, Kühl & Jørgensen 1994). Due to the scattering of light, surface maxima of photon scalar irradiance ranging from 180% to 280% of incidence irradiance are found in sediments, microbial mats and biofilms (e.g. Jørgensen & Des Marais 1988, Lassen et al. 1992, Kühl et al. 1994, Kühl & Jørgensen 1994). Kühl et al. (1994) found higher values of surface maxima in fine sediments (280% in the finest sediments of <63µm grain size), where scattering was more intense, compared to coarser sediments (180% of incidence irradiance at a grain size of 250 - 500µm). Increased scattering intensity in fine grained sediments results in a higher surface maximum of scalar irradiance. But at the same time, scattering enhances the probability of absorption, as absorption is enhanced at each encounter with a sediment particle, which results in higher attenuation of light in the fine grained sediments.

Regarding light as factor influencing mixotrophic feeding behavior, only phagotrophic activity of mixotrophic phytoflagellates at S1 and S2 matched my expectations. S1 exhibited the coarsest sediment. Oxygen production decreased with depth after a surface maximum, but the scattering of light was apparently not intense enough to completely attenuate it and oxygen was still produced even at 10mm depth, indicating that light was not limited for all photosynthetic species. These findings were also reflected by the taxonomic composition; chrysophytes, which are mostly phototrophic, still contributed 20% to the total flagellate community in the deepest sediment layer. MNF contributions to the total phytoflagellates

hardly varied with sediment depth in spite of decreasing light intensities. However, light intensities must have been still sufficient for some organisms to photosynthesize and might not have been low enough to induce increased phagotrophic activity in the mixotrophic community. Absolute abundances of MNF increased from the surface to the intermediate layer, as all species abundances except for bacteria did. This can possibly be explained by the fact that water movement directly behind the shoreline at S1 washed organisms out of the sediment surface layer, or organisms migrated into deeper layers to escape from resuspension.

At S2, I also found a surface maximum of light intensity and oxygen production, but light was absorbed more quickly due to increased scattering intensity in the finer grained sediment. Light did not propagate as deeply and photosynthetic oxygen production decreased much steeper than at S1, resulting in oxygen depletion at a depth of 5mm. Phagotrophic activity of MNF increased with depth and more MNF with ingested FLB were found in the intermediate sediment layer. MNF also contributed higher portions to both phytoflagellates and total grazers compared to the surface layer. Obviously, MNF were able to handle decreasing light intensities by increasing phagotrophic activity and thus to find a niche in that particular sediment layer. Beyond the euphotic zone, which is assumed to be within the oxygenated sediment here, MNF abundances decreased again. Photosynthesis is completely light limited here and only inactive photosynthetic or heterotrophic species can be found and only active mixotrophs that can survive completely on phagotrophy. Chrysophytes, which are known to have a great number of mixotrophic species (e.g. Boraas et al. 1988, Sanders & Porter 1988), were present with significant contributions (20%) in the surface and the intermediate sediment layer, but decreased in the deepest layer. This could be an indication for decreasing contributions of MNF in the deepest sediment layer.

At S3, the euphotic zone reached a depth of only 4.5mm. Due to the small sediment grain size, the light was scattered more effectively, which resulted in a more pronounced surface maximum of light and thereby oxygen production than at S2 on one hand. On the other hand, light was absorbed by scattering more quickly beyond the surface maximum of 2mm, and oxygen saturation decreased with a very steep gradient. Despite obvious light limitation, mixotrophic abundances decreased with depth and contributions to phytoflagellates hardly changed. A number of plankton studies have demonstrated that mixotrophic occurrence and feeding behavior depends on a number of factors, such as light, nutrients, prey abundances etc. (e.g. Sanders et al. 1990, Jones et al. 1993, Rothhaupt 1996a, Sanders et al. 2000). Light limitation was found to have disparate effects on different mixotrophic species (Caron et al. 1993, Skovgaard 1996, Hansen & Nielsen 1997, Stoecker et al. 1997, Holen 1999) and many

field studies have failed attribute phagotrophic feeding activity of mixotrophs to light limitation, as well as laboratory studies on particular mixotrophic species have failed to show increasing clearance rates at low light intensities (e.g. Bird & Kalff 1987, Arenovski et al. 1997). The species composition changed considerably from S2 to S3, and thereby mixotrophic community structure might have changed comparably. Physical and chemical sediment characteristics in a specific habitat result from an array of interacting biotic and abiotic events (Starink et al. 1996). Therefore, abundances of MNF can probably not be attributed to only one particular sediment characteristic such as light intensity. Other factors can be more crucial in determining mixotrophic feeding behavior, many of which are still unknown (Sanders et al. 2000).

The present study supports the fact that mixotrophy is an extremely variable phenomenon, which is difficult to be attributed to particular factors. This was also evident in the experiments described in Chapter 3, where light and nutrient conditions had disparate effects on mixotrophic communities in different experiments. Uncertainty in the factors inducing phagotrophy in different algal species makes it difficult to predict where and when mixotrophy will be an important ecosystem process (Sanders et al. 2000). Overall, mixotrophic abundances and contributions varied along the vertical and horizontal gradient of sediment characteristics, but in a range that was much smaller compared to previous plankton studies (e.g. Arenovski et al. 1995, Havskum & Riemann 1996, Safi & Hall 1999). Mixotrophs contributed minor portions to the total NF and negligible portions to the total grazers in these flagellate communities, which were all dominated by heterotrophs. They contributed somewhat to phytoflagellates (5-10%), but altogether, the mixotrophic feeding strategy played a minor role in microbial communities of the sediments investigated at this time of the year.

The experiments conducted so far in different sediments and seasons were restricted to Falckenstein Beach in the Western Baltic Sea. Variable patterns were found, but always with minor mixotrophic contributions. In order to estimate whether the patterns found in sediments so far can be generalized for coastal sediments or can only be attributed to the particular system considered so far, the significance of mixotrophs was investigated in different sediments and the overlying water column in contrasting systems at different geographical sites, described in the following Chapter 5.

# Chapter 5

## Occurrence and ecological impact of mixotrophic flagellates in coastal marine sediments: comparison of contrasting ecosystems at different geographical sites.

### 5.1. Introduction

Mixotrophic protists have been reported from a variety of plankton communities in different marine and freshwater environments (Sanders 1991b), where their potentially important contribution to community photosynthesis and predation has been demonstrated (Hall et al. 1993, Nygaard & Tobiesen 1993, Havskum & Riemann 1996, Havskum & Hansen 1997, Stoecker 1998, Safi & Hall 1999, Sanders et al. 2000). Their quantitative and qualitative impact within the microbial food web was found to be extremely variable on small temporal and spatial scales. Factors regulating the relative importance of photosynthesis and phagotrophy in mixotrophic algae, include light, nutrient availability, bacterial densities etc. Uncertainty in many factors inducing phagotrophy in different algal species still leads to difficulties in predicting where and when mixotrophy will be an important ecosystem process. In the sediments investigated so far, mixotrophs also showed variable contributions to phytoflagellates and bacterivores, but their overall abundance was much smaller than in many of the previous plankton studies noted above. FLB tracer experiments conducted hitherto (see Chapters 2-4) were restricted to sediment of one location in Kiel Fjord in the Western Baltic Sea (Falckenstein Beach, 15-21psu), where different sediment types were investigated (see Chapter 4). In the Baltic Sea, the salinity of water is the ultimate control of faunal and floral composition (Remane & Schlieper 1958, Segerstråle 1969). The salinity decline from the Southwestern to the Northeastern Baltic Sea constitutes almost 30psu. With decreasing salinity the diversity of marine species declines. This becomes most evident in the Central Baltic Sea with a salinity of 5-12psu. Here, we also find a minimum of freshwater species, since most of them are osmotically limited to a salinity of maximal 3psu (Rheinheimer 1993). Freshwater species are common in coastal areas with a strong freshwater influx as well as in the Northern Bottenwiek and in the Eastern part of the Gulf of Finland. This leads to a minimum of species diversity at a salinity of 5-7psu (Remane & Schlieper 1958).

Mixotrophic feeding behavior provides a number of potential benefits, such as survival on photosynthesis when prey concentrations limit heterotrophic growth (Andersson et al. 1989, Caron et al. 1990), providing a carbon/energy supplement under light-limited conditions by phagotrophy (Jones & Rees 1994a, Veen 1991) or increasing access to essential growth substances like phospholipids (Kimura & Ishida 1985). Despite these various benefits of a mixotrophic nutritional strategy, most protist taxa are specialized phototrophs or heterotrophs and are not mixotrophic. Clearly, a mixotrophic strategy exacts costs, which have been less thoroughly investigated than the potential benefits (Jones 2000). Raven has attempted to quantify the costs of mixotrophy (1995, 1997) and estimated that the photosynthetic apparatus and the machinery for uptake and assimilation of other inorganic nutrients besides carbon can account for up to 50% of the energy, C, N, P and Fe costs of cell synthesis for a phototrophic protist, whereas the phagotrophic apparatus was estimated to be <10%. These estimates imply that the cost for a primarily phototrophic protist of retaining a phagotrophic capability is relatively low, whereas the cost to a primarily phagotrophic heterotroph of maintaining phototrophic capability is rather high.

Taking the osmotic stress of marine species in low saline brackish water of the Baltic Sea into account, it can be assumed that the mixotrophic feeding behavior is a very costly strategy for many species. Low abundances of mixotrophic species were found in the sediments of Kiel Fjord (15-21 psu) so far and it can be assumed that mixotrophy plays a more important role in fully marine or freshwater sediments, where organisms are not subject to physiological constraints regarding salinity.

Furthermore, microbial community composition is likely to change with sediment structure and physical, chemical and biological interactions in different systems, where mixotrophy could be of higher importance within the microbial food web. The present study investigates whether previously found patterns in sediments of Falckenstein Beach have a general significance for coastal sediments or can only be attributed to characteristics of the particular system in Kiel Fjord. Therefore, three different fully marine sites were investigated in the Pacific Ocean in Southern California in March 2002 (Table 5.1.), where FLB grazing experiments were conducted to quantify mixotrophic protists. In addition, a set of five grazing experiments was conducted in late summer (September) of 2002 in different systems along a salinity gradient in Northern Germany, including a freshwater lake, three locations in the Baltic Sea and one in the North Sea (see Table 5.1.). At all sites, sediment and overlying plankton were sampled for FLB grazing experiments to compare mixotrophic contributions to both benthic and pelagic microbial communities.

| experiment | location | position | date | salinity (psu) | sampling | water depth (m) | treat-ments |
|---|---|---|---|---|---|---|---|
| **Exp.$I_{NB}$** | Newport Beach, Southern California, Pacific Ocean | 117° 55' 28''W 33° 36' 16''N | 07/03/2001 | 34 | sediment plankton | 0.5 | light/dark |
| **Exp.$II_{HB}$** | Huntington Beach, Southern California, Pacific Ocean | 118° 0' 43''W 33° 39' 46''N | 07/03/2001 | 29 | sediment plankton | 0.5 | light/dark |
| **Exp.$III_{Cat}$** | Catalina Island, Southern California, Pacific Ocean | 118° 29' 03''W 33° 26' 40''N | 26/03/2002 | 37 | "mud" "sand" plankton | 3 | light/dark |
| **Exp.$IV_{Falck}$** | Falckenstein Beach, Kiel Fjord, Western Baltic Sea | 10° 11' 40''E 54° 24' 23''N | 15/09/2002 | 21 | sediment ("shallow") sediment ("deep") plankton | 0.5 2.5 | light/dark |
| **Exp.$V_{Laboe}$** | Laboe, Kiel Fjord, Western Baltic Sea | 10° 13' 41''E 54° 24' 43''N | 14/09/2002 | 21 | sediment plankton | 0.5 | light/dark |
| **Exp.$VI_{Poel}$** | Western Baltic Sea | 11° 24' 37''E 54° 0' 30''N | 25/09/2002 | 11 | sediment | 2 | light/dark |
| **Exp.$VII_{Lake}$** | Schöhsee, Plön, Northern Germany | 10° 26' 40''E 54° 13' 08''N | 29/09/2002 | | sediment plankton | 0.5 | light/dark |
| **Exp.$VIII_{Syit}$** | Wadden Sea, North Sea | 8° 25' 56''E 55° 01' 29''N | 10-11/09/2002 | 31 | sediment (low tide) sediment (high tide) plankton | 0.5 | light/dark |
| **Exp$IX_{Ice}$** | Greenland Sea | 11° 23' 70''E 74° 33' 95''N | 19/07/2002 | 32 | brine and plankton | | light/dark |

**Table 5.1.** Experiments at all sampling sites (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Syit}$ and Exp. $IX_{Ice}$ Greenland Sea)

Furthermore, a completely different system was investigated: a microbial sea ice community of an ice floe in the Greenland Sea in comparison to oceanic plankton. Sea ice is an important structural element of polar marine ecosystems (Horner et al. 1992, Thomas & Diekmann 2002). Unlike freshwater ice, frozen seawater consists of a semisolid matrix, which is permeated by a network of channels and pores, varying in size from a few micrometers to

millimeters. These channels and pores are filled with brine, which is formed from expelled salts as the ice crystals freeze together (Eicken 1992) and in which viruses, bacteria, algae, protists, flatworms and small crustaceans live. Sea ice is dominated by strong gradients of temperature, salinity, space and light (Thomas & Dieckmann 2002, Krembs et al. 2002). These properties as well as the morphology of the brine channel system are highly variable and are determined by air temperature and snow cover. Sea ice properties differ seasonally and even diurnally, with small-scale variations in ice morphology, which is amplified by rafting of ice floes and deformation. This imparts a tremendous spatial heterogeneity to any sea ice zone also within a single floe. Due to the closed or semiclosed pore system within the ice, diffusion rates of dissolved gases and exchange of inorganic nutrients are greatly retarded (Gleitz et al. 1995). These sea ice characteristics imply that the mixotrophic feeding strategy could be an important survival strategy in this heterogeneous and variable system, since mixotrophs have been shown to change their nutritional mode in dependence of environmental factors such as light or nutrients (e.g. Sanders et al. 1990, Jones et al. 1993, Rothhaupt 1996a). Therefore, tracer experiments were conducted with brine communities and ambient plankton communities to estimate the potential significance of phagotrophic activity in phytoflagellates.

This study provides records of mixotrophic abundances in a variety of different benthic and pelagic coastal systems at different geographical sites, allowing to draw more general conclusions about the importance of mixotrophic flagellates in coastal marine ecosystems and to relate mixotrophy to salinity with regard to physiological constraints and energetical costs of mixotrophy in the Baltic Sea.

## 5.2. Material and Methods

### 5.2.1. Experiments and sampling sites

Three FLB grazing experiments were conducted in March 2002 at fully marine locations in the Pacific Ocean in Southern California (Table 5.1.). Huntington Beach and Newport Beach are located approximately 50km south of Los Angeles. Both locations are fully marine, but Huntington Beach is influenced by a freshwater stream, meeting the coastline at this point, resulting in a lower salinity than Newport Beach. The third experiment was conducted in a shallow bay on the northwest coast of Catalina Island, which is located 32km off the coast of LA. In the bay, two different sediment types were found; the coarser sediment is called "sand"

and the finer one "mud". In the following, the experiments are called Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach) and Exp.$III_{Cat}$ (Catalina Island). A set of five experiments was conducted in September 2002 along a salinity gradient, ranging from freshwater to a salinity of 31psu (Table 5.1.). Later in the summer, three locations were investigated in the Baltic Sea, Falckenstein Beach (see Chapters 2-4), Laboe at the eastern side of Kiel Fjord on the transition to the open Baltic Sea and a location 2km off the coast of Poel, a small island in the Bay of Mecklenburg. A fully marine location was sampled in the Wadden Sea at the North Sea island Sylt (31psu), on the coast of the most northeastern bay called Königshafen. This location is subject to strong tidal movements and sediment was sampled on low tide and high tide, where also the overlying water was sampled. The last experiment in September was conducted with sediment and plankton from the Schöhsee, a mesotrophic freshwater lake near Plön in Northern Germany. In the following, the 5 experiments conducted in September are called: Exp.$IV_{Falck}$, Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ and Exp.$VIII_{Sylt}$. In July 2002 one experiment was conducted on the Research Vessel *Polarstern* in the Greenland Sea (Table 5.1.). At this latitude in the Arctic summer, water and sea ice were subject to 24h of daylight. Sea ice (floe) and plankton were sampled and FLB grazing experiments were conducted on the ship. This experiment is called Exp$IX_{Ice}$.

In all experiments (I - IX), part of the sediment (or brine) and plankton were incubated in the dark and the other part was subjected to a light/dark cycle of 16:8 h.

### 5.2.2. FLB preparation

FLB were isolated from the particular sediments and the water column, which were investigated in the experiments, respectively, one week before the actual experiment was conducted. Bacteria were enriched with TSB and stained with DTAF as described in Chapter 2 to obtain monodispersed natural FLB, approximating the natural prey spectrum of nanoflagellates to greatest possible extent. Only for Exp$IX_{Ice}$, it was not possible to prepare FLB from the Greenland Sea before conducting the experiment. In this case, I used FLB prepared in advance from planktonic bacteria from Kiel Fjord.

### 5.2.3. Sampling procedure

*Sediment and plankton*

In Experiments I to VIII, sediment was sampled in the same way as described for Experiment I and II in Chapter 2. Sediment cores (acrylic glass) were taken with 4 replicates for each treatment and census, resulting in a total of 16 cores (light/dark, t0/t16). Sampling

had to be destructive, because it was not possible to take quantitative subsamples out of the sediment incubations. As in previous experiments, the shorelines were divided into 20 patches (1m wide) and 4 replicates were taken out of 4 randomly chosen patches to account for the heterogeneity of the system. Additional cores were taken to determine bacterial abundances for calculation of FLB concentrations to be added to the experiment (al incubations). Furthermore, surface sediment was collected for sieving analysis and determination of organic matter, as well as overlying water for nutrient analysis. Water samples were collected in 20l polyethylene containers. All samples were directly transferred to the laboratory where further processing took place (see Chapter 2).

Sediment - Sediment cores were extruded in the laboratory and the first 3mm of surface sediment, approximating a volume of 1.5cm³ +/- 0.4 cm³, were transferred into wells of tissue culture plates (see Chapter 2), which contained 2ml of sterile filtered seawater (SSW) of the different locations, respectively. After determination of sediment density, each sediment slice was weighed to calculate the accurate sediment volumes for each sample. A total of 12 experimental incubations were established per site; 4xt0, 4xt16 light and 4xt16 dark. Abundances of bacteria were determined and FLB were added in concentrations equal to 25% of the respective natural bacterial abundances. T0 samples were fixed with ice-cold glutardialdehyde at a final concentration of 1%. Dark incubations were covered with aluminum foil and all t16 samples were incubated in the climate chamber at 18°C with a light/dark cycle of 16:8h and a light intensity of 60µE $m^{-2} s^{-1}$. After an incubation time of 16h, t16 samples were fixed and all samples preserved at 4°C until further processing.

Plankton - Plankton was filtered through a 200µm gauze to exclude meso- and macrozooplankton species occurring in low abundance and to avoid uneven grazing in different bottles due to uneven species distribution in the 1l bottles used for the experiments (see Chapter 2). Then the samples were immediately filled into 1l polycarbonate bottles, with 4 replicates per treatment, respectively. Sampling was not destructive and t0 and t16 samples were taken out of the same bottles, resulting in a total of 8 bottles per site (4xlight, 4xdark). Natural bacterial abundances were determined and FLB were added (25% of the natural bacterial abundances). After that, a subsample of 27ml was fixed (t0) for all replicates with 3ml of 10% ice cold glutardialdehyde and stored at 4°C. Afterwards, the bottles were filled up with SSW. Dark incubations were incubated in closed boxes and all samples were incubated in a climate chamber under the same conditions as described for sediment samples. After 16h, t16 samples were taken in the same way as t0 samples and all samples were preserved at 4°C.

*Sea ice and plankton*

Sea Ice - The sea was covered approximately 10% by first year sea ice, being 2-4m thick, having a lot of snow cover and ridges, but no sediment loads. Ice floes varied in size from 10-100 m length. It was not possible to take ice cores out of big ice floes, therefore I had to crush a floe with the ship by driving through it and then collecting freshly broken pieces, where organisms were not washed out of the brine channels yet, with a large box (2m x 2m x 2m). Since it was not possible to ascertain on which particular part of the floe the ice chunks were located (i.e. the top or the bottom side of the floe), I used the whole ice block. Chunks of ice were processed on deck at *in situ* temperature (-2°C). For investigation of microorganisms in sea ice it is necessary to convert ice into a liquid phase, which is also necessary for FLB grazing experiments to allow for even FLB distribution. Big ice blocks were broken up on deck and mashed in sterile Whirlpak-bags (Nasco, volume: 1liter). In order to prevent losses due to osmotic shock, ice microorganisms were extracted from the brine contained within pore spaces by shaking an equal mixture of hand-mashed ice and filtered seawater (<0.2μm), used as salinity buffer. The liquid phase was pooled from all ice chunks and also filled into 2.7l polycarbonate bottles. This procedure was found to substantially reduce osmotic shock during the melting of ice cores and allows the recovery of up to 323% more protist cells than the traditional melting method (Sime-Ngando et al. 1997). Remaining crushed ice was pooled and melted with a surplus of SSW (2:1) to determine the number of remaining organisms and estimate the effectiveness of "washing out" the organisms from the brine channels. Volumes of added SSW and the melted sea ice remainder were noted to be able to reference determined abundances of organisms on ml brine.

Plankton - Surface seawater was collected with a bucket from the ship and immediately filled into 2.7l polycarbonate bottles, which were not filled completely at first to allow for thorough mixing after FLB addition. The incubation bottles were stored outside on deck to maintain *in situ* temperatures and light conditions.

Since t0 and t16 samples could be taken out of the same bottles, a total of 8 incubation bottles was established (4xdark, 4xlight) for brine and plankton, respectively. In addition, plankton and brine subsamples were frozen in 50ml polycarbonate centrifuge tubes for subsequent nutrient analysis (see below). Natural bacterial abundances were determined in both sea ice and plankton and then FLB were added approximating 25% of the natural bacterial abundances. After mixing, the bottles were filled up completely with plankton and brine/SSW mixture, respectively, and t0 samples were taken as described for plankton in section *Sediment and plankton* (see above). All bottles were filled up with SSW and dark

incubations were completely covered with aluminum foil. Bottles were incubated in on-deck incubators cooled by flow-through of surface water to assure *in situ* water temperatures and light conditions. After 16h incubation, t16 samples were taken in the same way as t0 samples. Fixed samples were preserved at 4°C and processed within 2 days after fixation.

### 5.2.4. Sample processing

Samples were processed in the same way as described in previous chapters. Sediment samples were "washed out" of the cell wells, diluted with SSW and subsamples collected on black 0.2µm polycarbonate filters (Nucleopore Track-Etch Membrane, PC MB 25mm 0.2µm). Plankton and sea ice samples were filtered directly. All samples were stained with DAPI at a final concentration of 5 µg/ml for 5min. Filters were mounted on slides, sealed with paraffin wax and stored immediately at -20°C until microscopical analysis.

### 5.2.5. Counting

Filters for the determination of microbial abundances were counted with an epifluorescence microscope (Leica/Leitz DM RB) at 1000x magnification in the same way as described in previous experiments (see Chapter 2).

### 5.2.6. Chemical analyses

Samples of all systems and sites were filtered through acid washed Whatman GF/F filters and dissolved nutrients were analyzed using a SKALAR SCANPLUS SYSTEM autoanalyser.

Sediment structure (grain size composition) was determined by sieving analysis in addition to determination of organic matter, performed as described in Chapter 4.

### 5.2.7. Statistical analysis

For all experiments, a one factor ANOVA (light/dark) on abundances of mixotrophs was conducted. Normal distribution and homogeneity of variances were tested with a Chi-Square-Test and with a Bartlett-Chi-Square-Test, respectively. Posthoc tests were conducted with Tukey's HSD test. The relationship between salinity and relative contributions of MNF to the total nanoflagellates was tested using a Pearson's correlation.

## 5.3. Results

### 5.3.1. Sediment analysis

8 of the 10 sediments investigated consisted to a major extent of grains between 0.5 and 1mm. The "muddy" sediment on Catalina Island was the finest, followed by the deep sediment at Falckenstein Beach and Laboe, whereas the sediments near Poel and in the Schöhsee were the coarsest ones (Fig. 5.1.). All sediments had very low contents of organic carbon, ranging from 0.3% to 1.25%; the sediments on Catalina Island had the highest contents of organic carbon (4%) (Table 5.2.).

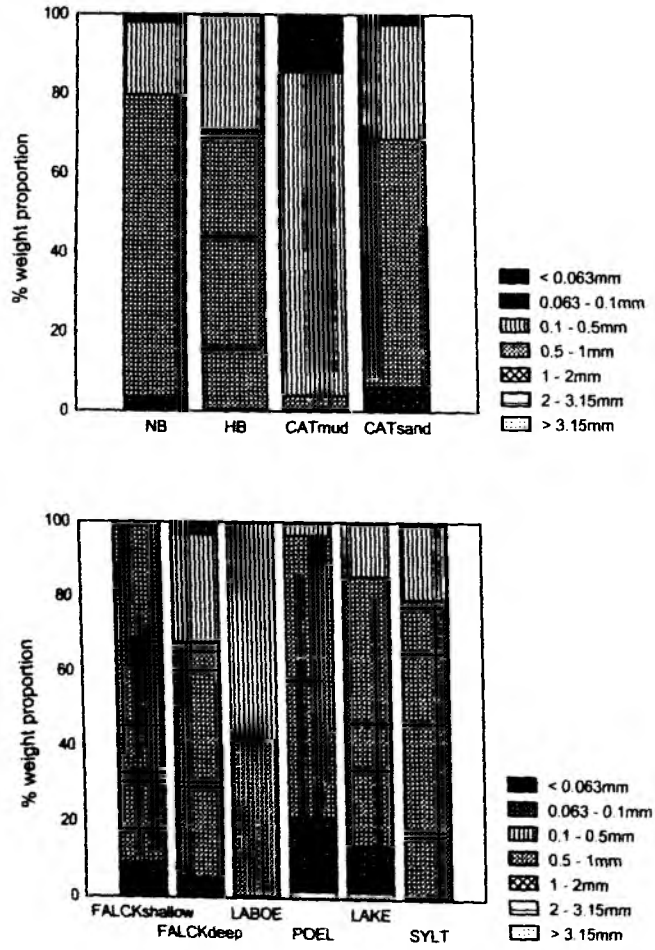



**Fig. 5.1.** Weight proportions of different size fractions of sediment grains in Experiments I-VIII (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Syl}$ ).

| | I | II | III$_{sand}$ III$_{mud}$ | IV$_{shallow}$ IV$_{deep}$ | V | VI | VII | VIII$_{low\ tide}$ VIII$_{high\ tide}$ |
|---|---|---|---|---|---|---|---|---|
| **% organic carbon** | 0.69 | 0.52 | 3.93<br>4.27 | 0.5<br>1.25 | 0.47 | 0.27 | 0.5 | 0.59<br>0.62 |

**Table 5.2.** Weight proportion of organic carbon, contributed to the total sediment for all Experiments I – IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee) and Exp.$VIII_{Sylt}$.

### 5.3.2. Nutrient analysis

The nutrient concentrations measured in overlying water at different sampling sites deferred considerably in dissolved nitrogen and phosphorus concentrations. Especially at Huntington Beach, extremely high concentrations of N and P were found (Table 5.3.), which can probably be attributed to the freshwater influx at that particular location. Concentrations were also high at Newport Beach, reaching 1/3rd of the nutrient concentrations at Huntington Beach. On Catalina Island, values for both N and P were much lower (Table 5.3.). In Kiel Fjord and on Sylt, N concentrations ranged from 0.5 to 4.5µmol/l and P concentrations from 0.1 to 1.3µmol/l (Table 5.3.), with lowest concentrations at Poel and in the Schöhsee. In the Greenland Sea, N and P concentrations in brine exceeded concentrations found in ambient water (Table 5.3.).

| | I | II | III. | IV | V | VI | VII | VIII | VIX$_{ice}$ | VIX$_{pl.}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **soluble N (sum of $NO_3^{2-}$, $NH^{4+}$)** | 31.18 | 9.76 | 0.68 | 3.54 | 7.47 | 0.45 | 0.46 | 2.18 | 4.00 | 1.57 |
| **soluble P** | 2.99 | 1.05 | 0.38 | 0.34 | 1.33 | 0.18 | 0.13 | 0.80 | 0.44 | 0.27 |
| **N : P** | 10.43 | 9.30 | 1.79 | 10.41 | 5.62 | 2.5 | 3.54 | 2.73 | 9.09 | 5.82 |

**Table 5.3.** Dissolved nitrogen N and phosphorus P at sampling sites of experiments I - IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee) and Exp.$VIII_{Sylt}$ and $VXI_{Ice}$).

### 5.3.3. Species analysis

In all sediment and plankton communities, PNF and HNF abundances exceeded MNF abundances by approximately one order of magnitude. In sediments of Newport Beach, Huntington Beach, Laboe, Schöhsee, Poel and shallow Falckenstein Beach, HNF, PNF, MNF and diatoms reached absolute abundances between $1x10^3$ and $5x10^5$, and bacteria between $1x10^7$ to $1x10^8$ (Fig. 5.2.). Abundances in Catalina sediments were slightly higher, in Sylt sediments and the deep sediment at Falckenstein Beach they were one order of magnitude higher. In all plankton communities, abundances of HNF, PNF, MNF and diatoms ranged from $1x10^2$ to $1x10^3$ (Fig. 5.2.) and of bacteria from $1x10^6$ to $1x10^7$; only in the Schöhsee, abundances did not exceed $1x10^5$. In the Greenland Sea, abundances of flagellates and diatoms were slightly higher in brine than in plankton communities, varying around $1x10^3$, only bacteria were slightly higher in plankton than in brine, ranging from $1x10^5$ to $1x10^6$ (Fig. 5.2.).

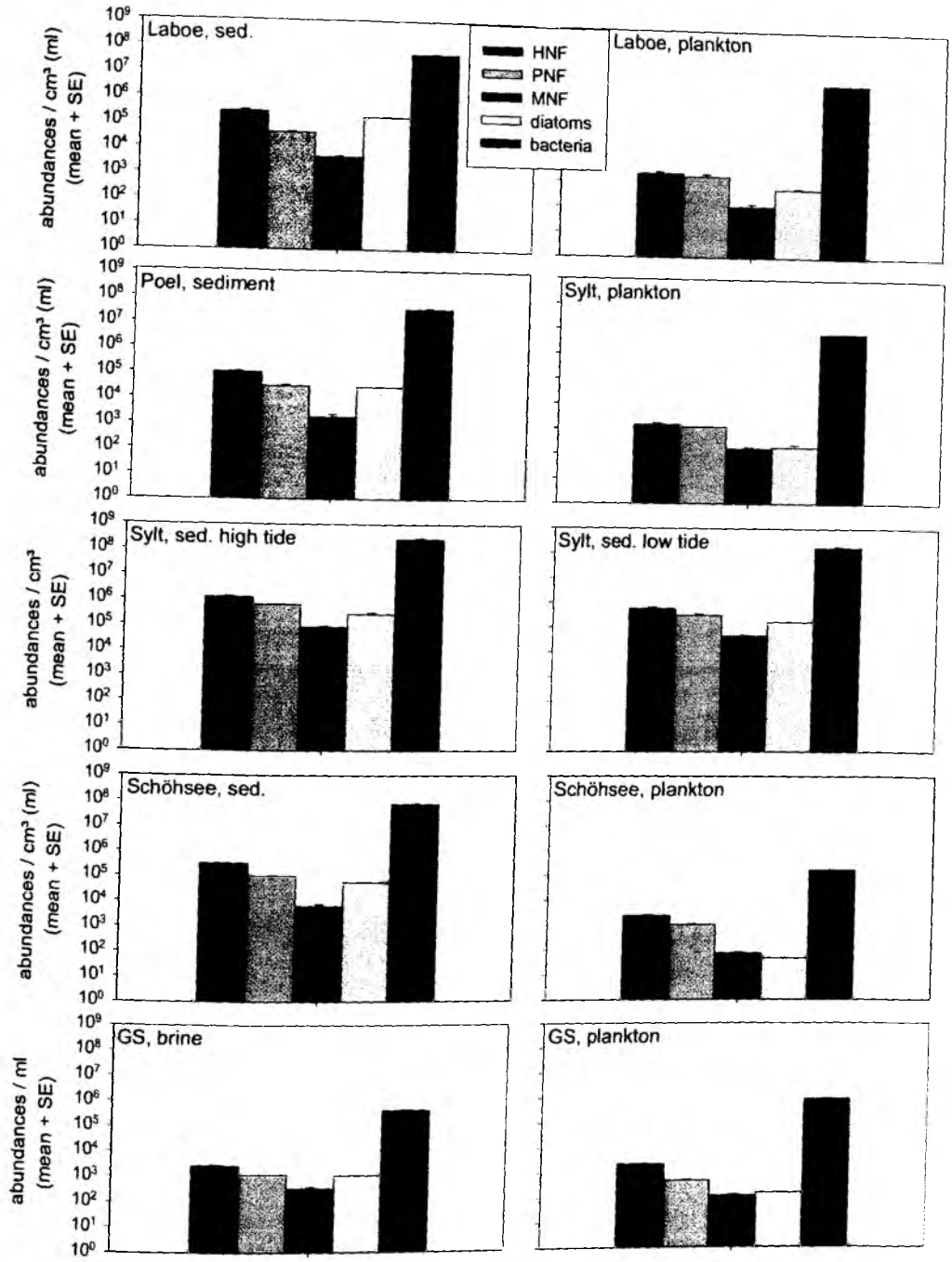



**Fig. 5.2.** Microbial community structure (abundances per $cm^3$ sediment) in sediment and plankton in light incubations of all Experiments I-IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea). Bars present absolute abundances of heterotrophic nanoflagellates (HNF), phototrophic nanoflagellates (PNF), mixotrophic nanoflagellates (MNF), diatoms and bacteria per $cm^3$ (or ml) sediment in dark incubations with natural $FLB_{nat}$.

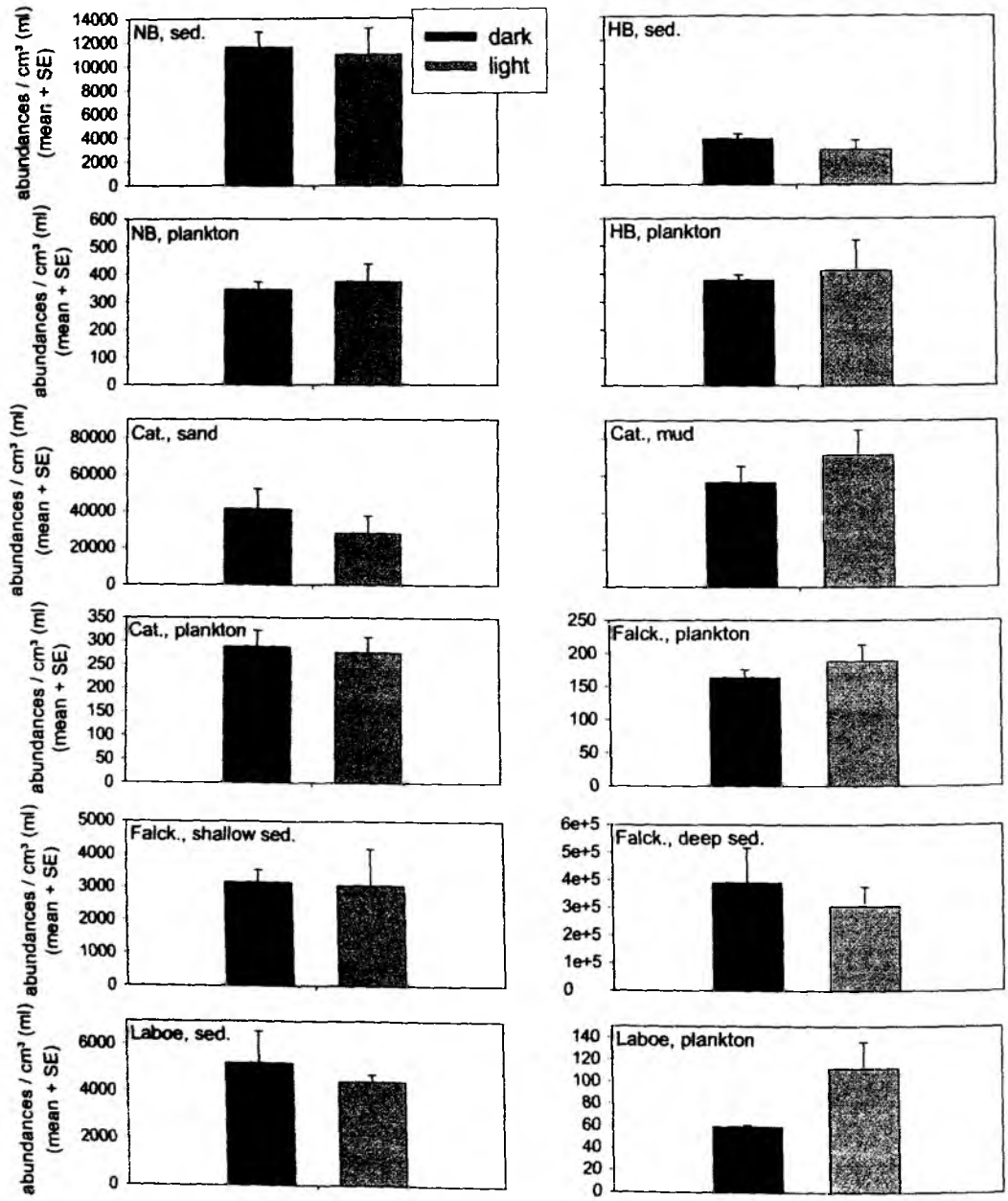



**Fig. 5.3.** To be continued

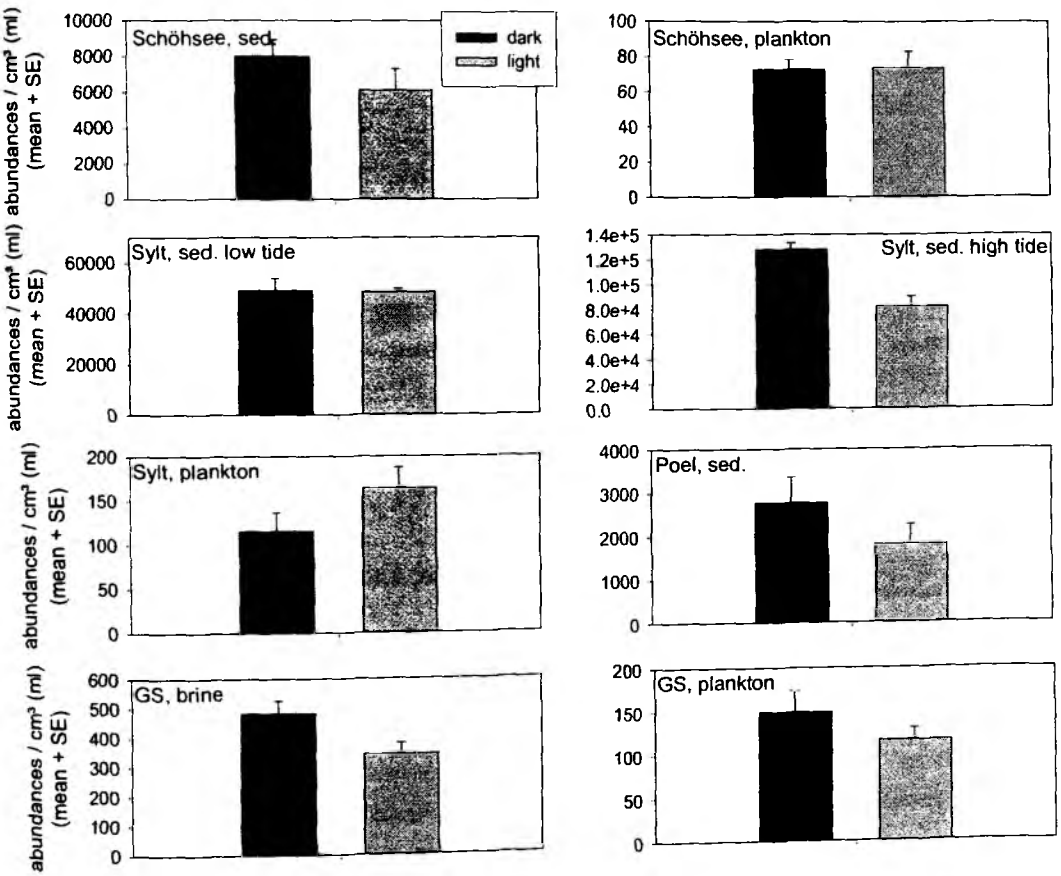



**Fig. 5.3**. Absolute abundances of mixotrophic nanoflagellates (MNF) per cm³ sediment (or ml plankton) in light and dark incubation in sediment and plankton of all experiments I-IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea).

Light and dark treatments did not affect mixotrophic feeding behavior in plankton communities at the coastal sites investigated. Phagotrophic activity hardly differed in light and dark incubations (Table 5.4., Fig. 5.3.). Only in the Greenland Sea, more MNF with ingested FLB were found in dark incubations in plankton (Fig. 5.3.), indicating increased phagotrophic activity; this effect was significant for brine ($p=0.045$, Table 5.4.). In most sediment communities, there was only a slight trend of higher phagotrophic activity in dark incubations, where more MNF with ingested FLB were found (Fig. 5.3.); this effect was only significant for sediment on Sylt at high tide ($p=0.003$, Table 5.4.). No effects were found in the Sylt sediment at low tide, as well as in sediments at Newport Beach, $Catalina_{mud}$ and $Falckenstein_{shallow}$.

| Factor | df | light/dark (1) |
|---|---|---|
| **Exp.$I_{NB}$ (sed.)** | 6 | 0.061 (0.813) |
| **Exp.$I_{NB}$ (plank.)** | 6 | 0.149 (0.713) |
| **Exp.$II_{HB}$ (sed.)** | 6 | 1.003 (0.355) |
| **Exp.$II_{HB}$ (plank.)** | 6 | 0.097 (0.766) |
| **Exp.$III_{Cat\text{-}sand}$ (sed.)** | 6 | 0.842 (0.394) |
| **Exp.$III_{Cat\text{-}mud}$ (sed.)** | 6 | 0.867 (0.388) |
| **Exp.$III_{Cat}$ (plank.)** | 6 | 0.059 (0.816) |
| **Exp.$IV_{Falck\text{-}shallow}$ (sed.)** | 6 | 0.006 (0.940) |
| **Exp.$IV_{Falck\text{-}deep}$ (sed.)** | 6 | 0.347 (0.578) |
| **Exp.$IV_{Falck}$ (plank.)** | 6 | 0.628 (0.473) |
| **Exp.$V_{Laboe}$ (sed.)** | 6 | 0.302 (0.602) |
| **Exp.$V_{Laboe}$ (plank.)** | 6 | 3.987 (0.117) |
| **Exp.$VI_{Poel}$ (sed.)** | 6 | 1.655 (0.246) |
| **Exp.$VII_{Lake}$ (sed.)** | 6 | 1.566 (0.257) |
| **Exp.$VII_{Lake}$ (plank.)** | 6 | 0.005 (0.948) |
| **Exp.$VIII_{Sylt\text{-}low\ tide}$ (sed.)** | 6 | 0.015 (0.907) |
| **Exp.$VIII_{Sylt\text{-}high\ tide}$ (sed.)** | 6 | 22.245 **(0.003)** |
| **Exp.$VIII_{Sylt}$ (plank.)** | 6 | 2.355 (0.176) |
| **Exp. $IX_{Ice}$ (brine)** | 6 | 6.407 **(0.045)** |
| **Exp. $IX_{Ice}$ (brine)** | 6 | 1.386 (0.284) |

**Table 5.4.** Results of a one factor ANOVA (light/dark) on abundances of mixotrophic nanoflagellates (MNF) for all experiments (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea). The table gives the F-ratios (with significance levels in parentheses) for the main factors for all experiments. The degrees of freedom for the effect terms are given in parentheses for the effect, for the error term in the first column of the analysis. Effects significant at $p<0.05$ are printed in bold, trends with $p<0.1$ are printed in italics. Results for Exp.$II_{HB}$ for plankton and for Exp.$V_{Laboe}$ should be considered with care since a violation of variance homogeneity was detected (Exp.$II_{HB}$: Bartlett's $\chi^2=5.35$, $p=0.021$; Exp.$V_{Laboe}$ (sediment): Bartlett's $\chi^2=4.462$, $p=0.035$; Exp.$V_{Laboe}$ (plankton): Bartlett's $\chi^2=5.164$, $p=0.023$).

The protist communities in sediments were dominated by HNF, contributing 60% to 80% to the total nanoflagellates (Fig. 5.4.). PNF only contributed around 20% to the total NF at Newport Beach, Huntington Beach, Laboe, Poel and in the Schöhsee, whereas they contributed 30-40% in sediments of Catalina Island, Falckenstein and Sylt and in the brine of sea ice. MNF occurred with lowest portions in the Schöhsee, at Poel and Laboe and had highest contributions to the total NF in sediments of Falckenstein Beach, Sylt and Catalina Island (4-6%) (Table 5.5., Fig. 5.4.). In plankton communities, PNF contributions were generally higher, ranging from 30% to 50%, except for the plankton in the Greenland Sea, where PNF only contributed 20% to the total NF (Table 5.5., Fig. 5.4.). However, MNF contributions in plankton communities hardly differed from respective sediment communities, ranging from 2% - 6.6 % (Table 5.5., Fig. 5.4.). Highest contributions of MNF were found in

the sea ice community in brine, where 10% of the total NF were mixotroph, whereas they contributed only 4% - 6% to the plankton community in the Greenland Sea.

| Experiment | % MNF dark | % MNF light | % HNF+FLB dark | % HNF+FLB light |
|---|---|---|---|---|
| $I_{NB}$ sediment | 4.4 ± 0.3 | 3.9 ± 0.8 | 13.3 ± 1.4 | 16.4 ± 2.2 |
| $I_{NB}$ plankton | 5.2 ± 0.3 | 6.0 ± 0.8 | 12.5 ± 0.9 | 13.0 ± 3.3 |
| $II_{HB}$ sediment | 4.9 ± 0.5 | 3.3 ± 0.3 | 18.9 ± 1.8 | 14.6 ± 1.4 |
| $II_{HB}$ plankton | 4.5 ± 0.4 | 5.7 ± 1.2 | 10.4 ± 1.1 | 7.7 ± 1.1 |
| $III_{Cat\text{-}sand}$ sediment | 6.5 ± 0.7 | 4.8 ± 1.0 | 15.6 ± 2.4 | 18.1 ± 4.7 |
| $III_{Cat\text{-}mud}$ sediment | 5.2 ± 0.4 | 7.5 ± 0.9 | 13.2 ± 2.2 | 18.8 ± 2.3 |
| $III_{Cat}$ plankton | 5.8 ± 0.6 | 6.0 ± 0.5 | 13.8 ± 1.2 | 11.7 ± 0.8 |
| $IV_{Falck\text{-}shallow}$ sediment | 5.7 ± 0.9 | 4.6 ± 0.8 | 21.2 ± 2.2 | 21.4 ± 3.5 |
| $IV_{Falck\text{-}deep}$ sediment | 4.8 ± 0.4 | 4.8 ± 0.3 | 15.9 ± 1.1 | 16.5 ± 1.7 |
| $IV_{Falck}$ plankton | 5.6 ± 0.4 | 4.7 ± 0.4 | 22.0 ± 2.5 | 16.2 ± 1.0 |
| Exp.$V_{Laboe}$ sediment | 2.2 ± 0.6 | 1.5 ± 0.1 | 9.1 ± 0.3 | 6.7 ± 1.1 |
| Exp.$V_{Laboe}$ plankton | 2.5 ± 0.1 | 2.8 ± 0.2 | 15.7 ± 2.5 | 14.8 ± 1.0 |
| Exp.$VI_{Poel}$ sediment | 1.4 ± 0.3 | 1.3 ± 0.3 | 4.7 ± 0.4 | 8.4 ± 1.8 |
| Exp.$VII_{Lake}$ sediment | 2.3 ± 0.3 | 1.4 ± 0.2 | 8.4 ± 0.3 | 8.0 ± 0.7 |
| Exp.$VII_{Lake}$ plankton | 2.3 ± 0.3 | 2.1 ± 0.3 | 16.9 ± 2.3 | 15.7 ± 1.0 |
| Exp.$VIII_{Sylt\text{-}low\ tide}$ sed. | 6.2 ± 0.7 | 5.4 ± 0.8 | 21.5 ± 0.8 | 21.5 ± 1.6 |
| Exp.$VIII_{Sylt\text{-}high\ tide}$ sed. | 6.4 ± 0.7 | 4.2 ± 0.5 | 27.1 ± 1.7 | 21.3 ± 1.4 |
| Exp.$VIII_{Sylt}$ plankton | 6.6 ± 1.0 | 5.7 ± 0.5 | 28.5 ± 2.6 | 21.7 ± 1.0 |
| Exp. $IX_{Ice}$ brine | 10.3 ± 0.7 | 8.0 ± 0.6 | 25.2 ± 0.4 | 27.4 ± 1.3 |
| Exp. $IX_{Ice}$ plankton | 5.9 ± 1.0 | 4.2 ± 0.5 | 23.6 ± 2.0 | 17.8 ± 0.9 |

**Table 5.5.** Relative abundances of mixotrophic nanoflagellates as % of the total nanoflagellates (%MNF / NF) and heterotrophic nanoflagellates with ingested FLB as % of the total heterotrophic nanoflagellates (%HNF + FLB / total HNF) for all Experiments I – IX (Exp.$I_{NB}$ (Newport Beach). Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea). Values present mean (n=4) ± standard error.

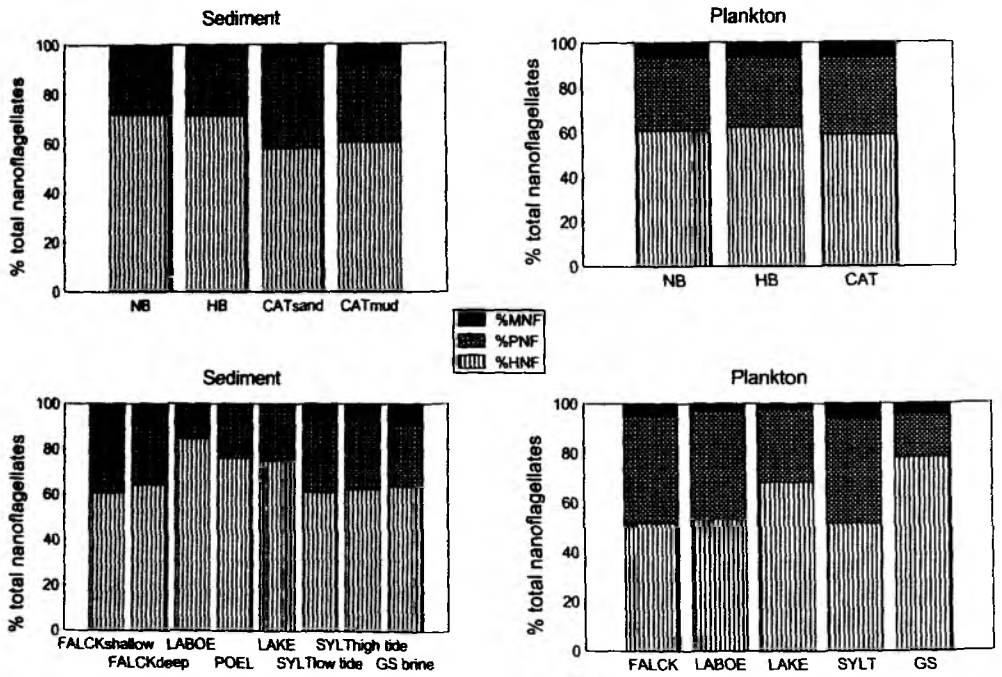



**Fig. 5.4**. Relative abundance of heterotrophic (HNF) phototrophic (PNF) and mixotrophic (MNF) nanoflagellates as percentage of the total nanoflagellates in sediment, brine and plankton of Experiments I-IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea).

In sediment and plankton communities in both light and dark incubations, MNF contributions to the total nanoflagellates significantly increased with increasing salinity (Fig. 5.5.), ranging from 2% in the Schöhsee and at Poel to 6-7% in at fully marine locations and even 10% in the brine of sea ice.

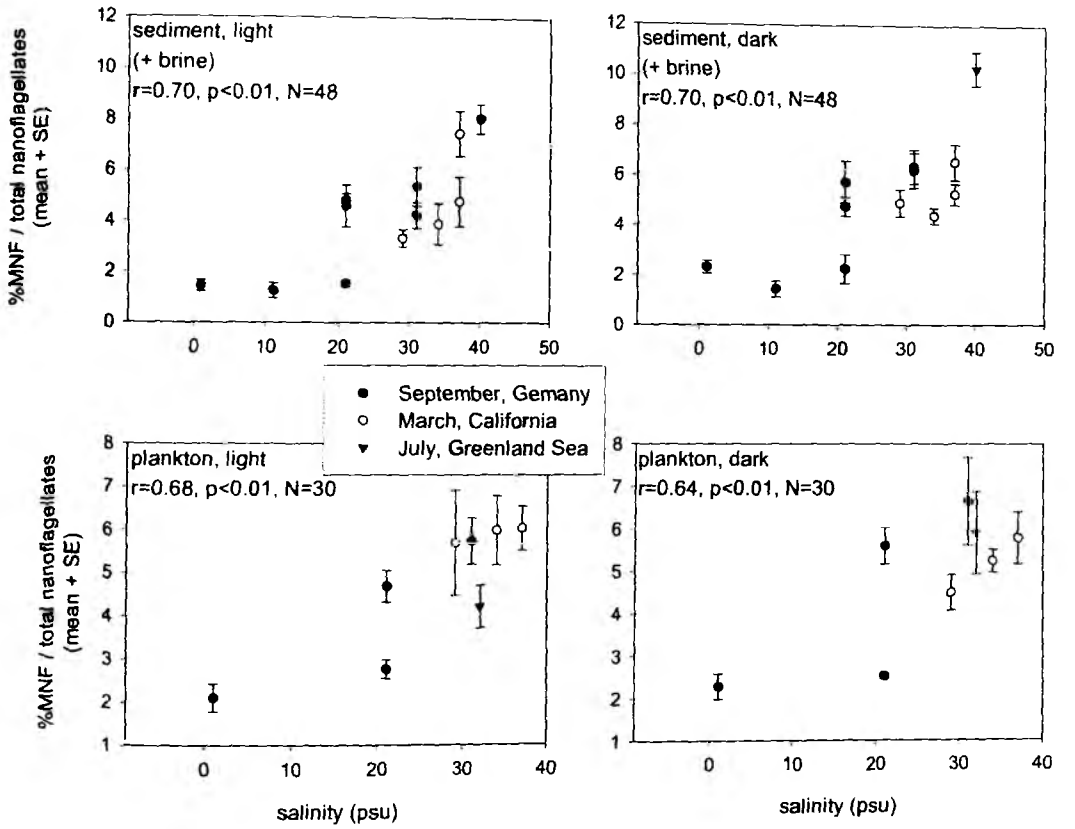
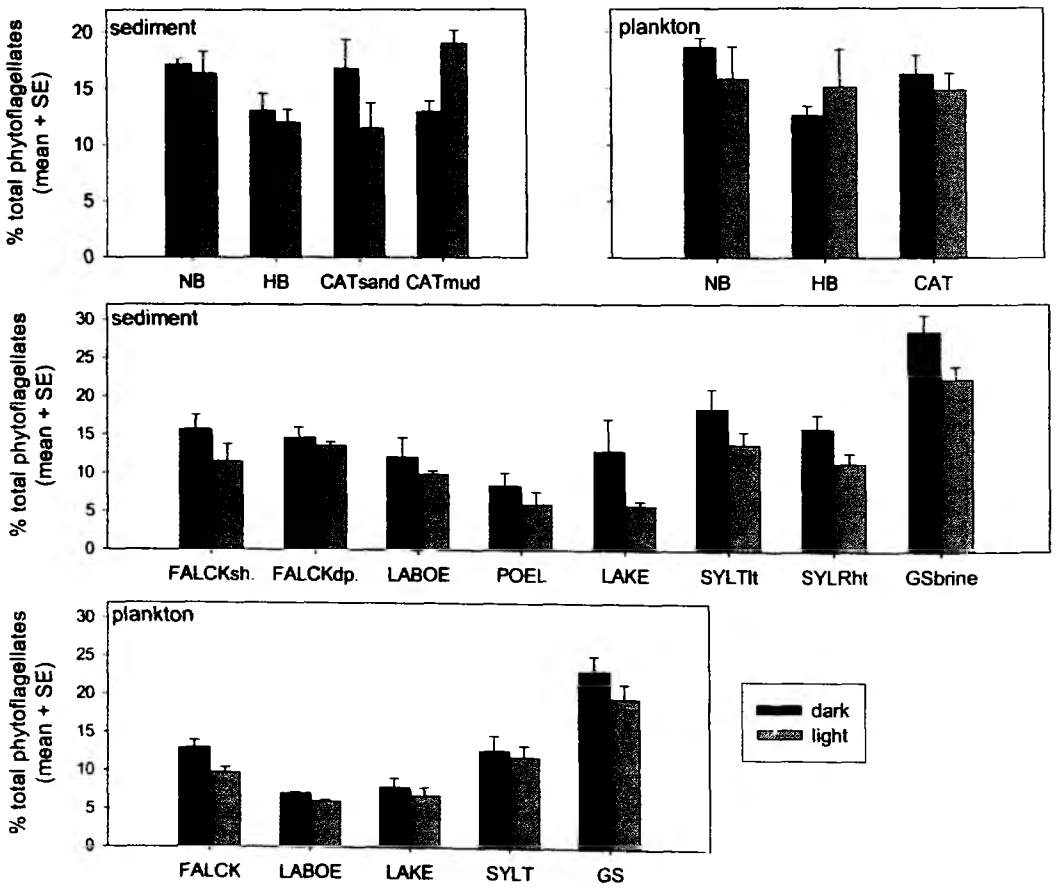



**Fig. 5.5.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total NF in all experiments I-IX (conducted in September 2002 in Germany, in March 2002 in California and in July 2002 in the Greenland Sea along the salinity gradient. Note the different scaling on the y-axes. Pearson's correlations are presented for all graphs as r = correlation coefficient, p = level of significance, N = number of values.

Relative abundances of MNF as % of the total PNF ranged from 5% to 20% in sediment and plankton communities at all sites, with lowest values in the sediment of Poel and in plankton communities of Laboe and the Schöhsee (Fig. 5.6.A). Highest contributions were found in the Greenland Sea, where MNF contributed 20-25% to the total PNF in plankton and as much as 30% in brine (Fig. 5.6.A). As bacterivores, MNF contributed 6-14% to the total grazers (HNF + MNF) in sediment and plankton communities with highest contributions at Catalina Island and Sylt (Fig. 5.6.B) and lowest in sediment and plankton of Laboe and the Schöhsee, as well as in sediment from Poel. Maximum contributions of almost 15% were found in the brine of sea ice in the Greenland Sea, being twice as high as in plankton contributions (Fig. 5.6.B).

(A)


sediment
plankton
% total phytoflagellates (mean + SE)
NB
HB
CATsand
CATmud
CAT
FALCKsh.
FALCKdp.
LABOE
POEL
LAKE
SYLTlt
SYLRht
GSbrine
FALCK
SYLT
GS
dark
light

(B)

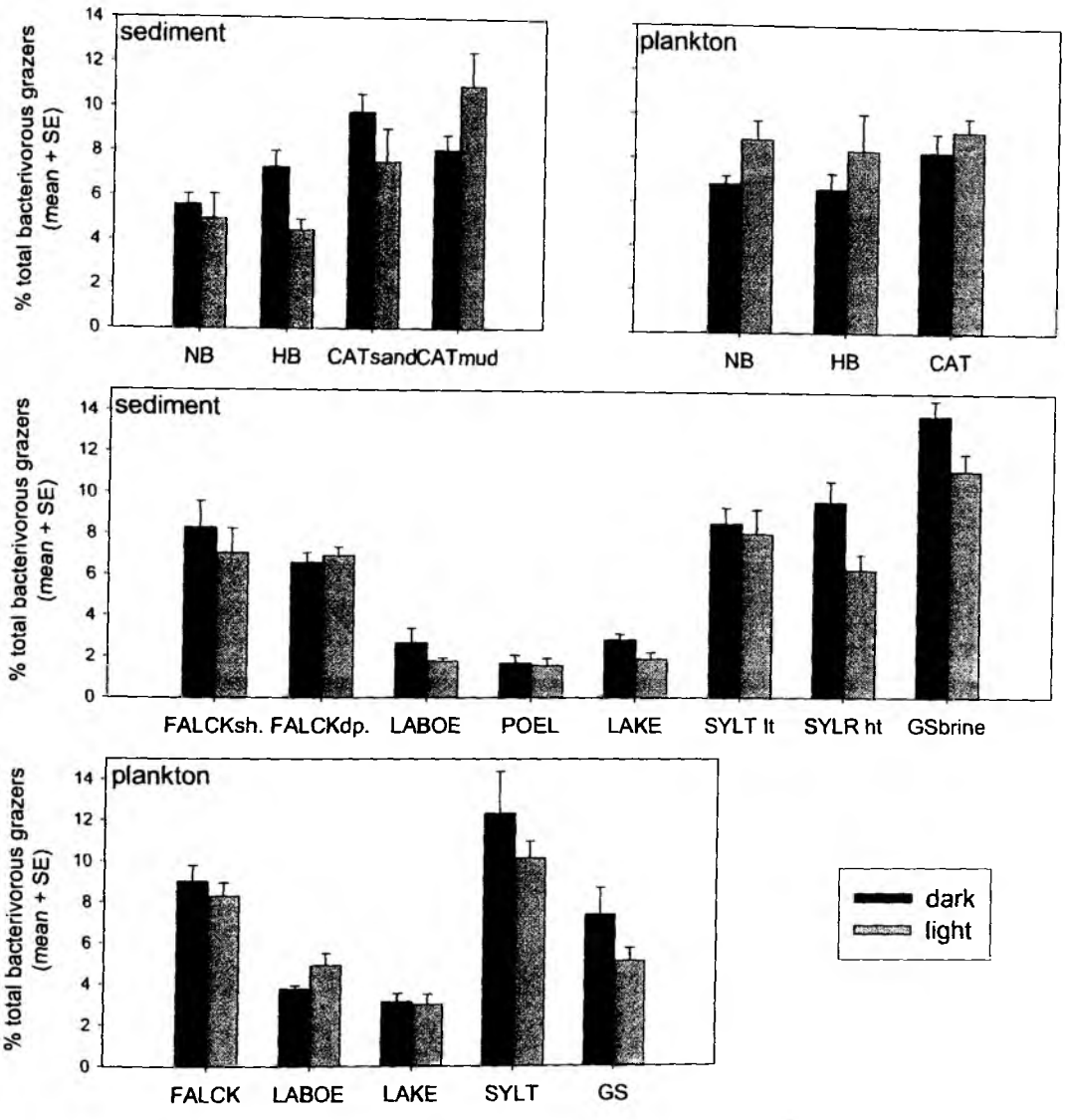



**Fig. 5.6.** Relative abundances of mixotrophic nanoflagellates (MNF) as percentage of the total phototrophic nanoflagellates (PNF) (A) and the total heterotrophic nanoflagellates (HNF) (B) in light and dark incubation in sediment and plankton of all Experiments I-IX (Exp.$I_{NB}$ (Newport Beach), Exp.$II_{HB}$ (Huntington Beach), Exp.$III_{Cat}$ (Catalina Island), Exp.$IV_{Falck}$ (Falckenstein Beach), Exp.$V_{Laboe}$, Exp.$VI_{Poel}$, Exp.$VII_{Lake}$ (Schöhsee), Exp.$VIII_{Sylt}$ and Exp. $IX_{Ice}$ Greenland Sea). Note the different scaling on the y-axes.

Phagotrophic feeding activity of HNF, i.e. HNF with ingested FLB as percentage of the total HNF was lowest in the sediment of Poel, where only 5-9% of the HNF ingested FLB (Table 5.5.) and highest in sediments and plankton of Sylt and Falckenstein and in brine and

plankton of the Greenland Sea, ranging from 17-27% (Table 5.1.). In remaining sediments and plankton, HNF with ingested FLB contributed 12-18% to the total HNF.

## 5.4. Discussion

In the sediment and plankton communities mixotrophs contributed maximum portions of 7% to the total nanoflagellates, which were only exceeded in the brine of sea ice. Mixotrophic contributions increased with increasing salinity, being lowest in the Schöhsee and in Poel and highest in fully marine locations like Catalina Island and in the high saline brine of sea ice. These results support the initial hypothesis that mixotrophy is a very costly strategy for many species, especially when subject to physiological constraints regarding salinity. The location at Poel with a salinity of 11psu is within the range of the lowest species diversity in the Baltic Sea, since marine species have their distribution limit below 12psu and freshwater species are osmotically limited to a salinity of maximal 3psu (Rheinheimer 1993). Despite all potential benefits, a mixotrophic feeding strategy exacts costs, which has been estimated by Raven (1995, 1997) to be especially high to a primarily phagotrophic heterotroph, maintaining phototrophic capability. My results indicate that the potential benefits derived from mixotrophy cannot countervail its energetical costs, given the effort for compensating physiological constraints (osmotic stress) in low saline brackish water. In the Schöhsee, freshwater species are free from physiological constraints regarding osmotic stress, and MNF contributions equal to marine communities in their fully marine habitats could be expected. However, MNF contributions were very low and fitted into the salinity gradient. Mixotrophs were shown to contribute significantly to freshwater plankton communities (Bird & Kalff 1986, 1987, Berninger et al. 1992, Jansson et al. 1996), contributing $\leq$10% to over 41% of the total phytoplankton. But mixotrophic contributions varied considerably in different seasons and on smaller temporal and spatial scales (Bird & Kalff 1986, 1987, Sanders 1991, Berninger et al. 1992, Bennett et al. 1996). Keeping in mind that results of this study only present "snapshots" of microbial communities at different sites at one particular time point and that only one freshwater habitat was investigated, it can be concluded that MNF can play a different role within the microbial food web under different circumstances.

The significance of mixotrophs has not only been shown to be very variable in freshwater habitats, but also in marine systems. Arenovski et al. (1995) found MNF to comprise 5-53% of the phototrophic nanoplankton in surface waters of the Sargasso Sea and Havskum & Riemann (1996) found mixotrophs to account for 9-49% of the phototrophic biomass in the

Bay of Aarhus (Denmark). In an extreme case, Safi & Hall (1999) found almost all identifiable species of PNF in the Pacific Ocean east of New Zealand's South Island to be capable of ingesting FLP (fluorescently labeled particles). Other studies demonstrated their significant role as grazers, where mixotrophic flagellates contributed up to 60% of the bacterivory and 57% of the herbivory on picophytoplankton and small (<5μm) nanophytoplankton (Hall et al. 1993, Nygaard & Tobiesen 1993, Havskum & Riemann 1996, Havskum & Hansen 1997, Safi & Hall 1999). Although very variable on temporal and spatial scales, these sometimes large proportions of MNF have important implications for algal nutrition, nutrient dynamics and food web interactions in planktonic ecosystems (Sanders et al. 2000). Mixotrophic contributions to total PNF or total bacterivorous grazers only varied in a range of 5-20% or 2-12%, respectively, in sediment and plankton communities at all sites investigated. Thus mixotrophs had a considerable potential to account for primary production, but a minor importance in controlling bacterial abundances. This study indicates that MNF are of minor significance in coastal sediments and in shallow coastal plankton compared to plankton deeper in the water column. In shallow coastal plankton communities, light is rarely a limiting factor for photosynthesis, since light propagates through the entire water column. Here, MNF did not respond at all to changing light conditions by showing increased phagotrophic activity when photosynthesis was light limited, indicating that mixotrophs do not have to be adapted to changing light conditions. In contrast to that, light is a limiting factor for photosynthesis in deeper water columns with a greater mixing depth, when organisms are carried into deeper water layers. Here, mixotrophy can be a very advantageous feeding strategy, allowing phytoflagellates to switch to phagotrophy at unfavorable light conditions.

The highest contributions of mixotrophs were found in the brine of sea ice, where MNF contributed 10% to the total nanoflagellates, accounting for 30% of the total PNF and 15% of the total bacterivorous grazers. In the ambient plankton of the Greenland Sea, mixotrophic contributions were lower, with MNF contributing up to 25% to the total PNF and 6-8% to total bacterivorous grazers. Mixotrophic feeding behavior can be an advantageous strategy for phytoflagellates living in brine channels of sea ice, which is subject to extreme variability of light, nutrients, salinity and other environmental factors. Sea ice, especially when covered with snow, is an effective barrier to light transmission, and sea ice algae have to be physiologically adapted to living at low light (Thomas & Dieckmann 2002). Palmisano & Garrison (1993) proposed sea ice algae to be able to switch from autotrophy to heterotrophic uptake of organic matter during periods of very low light, especially in winter, but their

evidence was inconclusive. Zaslavskaia et al. (2001) showed that a single gene encoding a glucose transporter can be introduced into photosynthetic marine diatoms to induce a fundamental change in metabolism, allowing the organisms to grow on glucose in the absence of light. Thomas & Dieckmann (2002) proposed that similar metabolic shifts occur in natural sea ice diatoms during changing environmental conditions. My study provides first data on the significance of mixotrophic flagellates in the brine channels of Arctic sea ice, indicating their potential importance in sea ice communities.

Mixotrophs responded to light limitation with increased phagotrophic activity in both plankton and sea ice communities. The ability of mixotrophs to switch from photosynthesis to phagotrophy under light limitation may be advantageous for them in both systems. In the brine channels, light conditions are influenced by snow cover, melting processes or collision of ice floes, changing the whole shape and structure of the floe and influencing light conditions. Therefore, it can be advantageous for MNF not only to pursue one particular trophic mode, but to be able to facultatively switch from photosynthesis to phagotrophy when light is limited. In addition, steep gradients of salinity, nutrients, pH etc. (see above) influence microbial community dynamics and composition. In such environments flagellates are likely to have an advantage when not only depending on one particular trophic mode but to have two ways to gain energy. In the plankton of the Greenland Sea it will also be a favorable strategy for mixotrophs to be adapted to changing light conditions since floating ice floes or broad ice cover shield the water column from light, thus impairing photosynthesis in plankton communities. Furthermore, organisms in both sea ice and plankton have to cope with extremely low light conditions during the Arctic winter, where phagotrophic nutrition might be an important survival strategy for mixotrophs.

In coastal plankton and sediments, MNF hardly responded to changing light conditions, although there was a slight trend of increased phagotrophic activity in sediments incubated in the dark. One could argue that sediments are characterized by steep vertical gradients of light, along which phytoflagellates move. Here, it could be indeed favorable for phytoflagellates to survive on phagotrophy in deeper sediment layers, especially during summer, when other phototrophs like diatoms can form dense mats in the sediment surface layer, where they compete for space and light. Given these arguments, it is difficult to understand why the mixotrophic feeding strategy was not found to play a major role in the investigated sediments. Features like steep physical and chemical gradients, which were proposed to influence mixotrophic feeding behavior in the brine channels of ice floes, should be as important in sediments. However, organisms that inhabit brine channels are plankton organisms, which

become trapped within the channels as surface water freezes and the ice grows and consolidates (Eicken 1992, Palmisano & Garrison 1993). They return to the water column as the ice melts. A completely different microbial community structure is given compared to sediments and it can be assumed that other mechanisms than steep gradients generate community structure and the importance of mixotrophs. In sediments, the organisms can freely move along vertical gradients and can ascent for instance towards better light conditions when light becomes limiting in deeper layers. This is not possible in the brine channels where species are trapped in a labyrinth of pores and have to cope with changing environmental conditions becoming unfavorable without being able to escape. Also in plankton communities with a great mixing depth, organisms can not escape changing environmental conditions such as decreasing light intensities, once they are carried into deeper water layers; they depend on being carried again to surface waters. Therefore it can be assumed that mixotrophic feeding strategies are essential for microbial communities in particular pelagic environments as well as in brine channels, whereas they only present a supplementary feature for organisms in sediments.

Overall, the significance of mixotrophy in sediments can not be explained by single factors. An array of different factors, interacting with mixotrophy on species level, population level and community level to different extents and on different time scales makes it almost impossible to predict where and when mixotrophy will be an important ecosystem process. However, mixotrophy can be an important mechanism and imparts additional complexity to energy production, elemental flow and trophic relationships among microorganisms. These aspects are further discussed in Chapter 6.

# Chapter 6

## General Discussion

The present study provides first records of the occurrence of mixotrophic protists and their ecological role in coastal sediments. Mixotrophic nanoflagellates were found in all sediments investigated, but overall, their abundances and contributions to the nanoflagellate communities and to community bacterivory indicated a minor role within benthic microbial sediment food webs compared to plankton communities in oceanic and freshwater environments (see Chapter 1).

### 6.1. Evolutionary aspects

Regarding the evolutionary context of mixotrophy, Raven (1997) emphasized that phagotrophy has been a crucial element in the evolution of photosynthetic eukaryotes. The origin of their plastids can be traced to one or more endosymbiotic events involving phagotrophic ingestion and retention of unicellular photosynthetic organisms. Therefore, phagotrophy must be considered as a primitive character and its absence in most photosynthetic protists a derived character. Raven (1997) pointed out that the additional cost of phototrophy in a mixotrophic protist can be up to 50% of the energy, carbon, nitrogen, phosphorus and iron budget of the cell, whereas the cost of synthesis and maintenance of the feeding apparatus in a mixotrophic protist has been estimated to be ~10%. Therefore, it would be more costly for a heterotrophic protist to be photosynthetic than for an algae to be phagotrophic. Photosynthetic eukaryotes show many independent examples of these endosymbiotic events, with up to three sequential uptakes of endosymbionts involved in the evolution of some eukaryotes. Therefore, the retention by phagotrophs of photosynthetic capability from ingested phototrophs must have arisen rather readily when the right selection pressures operated. A very large number of planktonic groups have independently evolved some form of mixotrophy (Jones 1994). It can be speculated that selection pressure events favored mixotrophic feeding strategies more in planktonic environments than in benthic environments like sediments. Selection pressure events might have included situations where phagotrophic nutrition was not sufficient for survival or when environmental factors such as light, nutrients, prey abundances etc., limited pure autotrophy or pure heterotrophy. Due to the input from land, nutrient availability should be a minor problem for photosynthetic species in

coastal sediments compared to oceanic plankton. Besides, bacterial densities in sediments are in general three orders of magnitude higher than in plankton communities. Abundances of flagellates are also higher in sediments than in plankton, but studies on benthic microbial food webs indicated that nanoflagellates are not able to control bacterial numbers most of the year (Starink et al. 1996b, Epstein 1997b). Hence, it can be assumed that bacterial prey abundances in sediments will rarely be limiting for heterotrophic flagellates. In sediments, bacteria are interstitial or attached to sediment particles, providing a great availability of different niches to occupy for bacterial grazers. Starink et al. (1994a) hypothesized that phagotrophic protist populations living in such environments have a wide range of feeding strategies in order to maximize niche segregation. This niche segregation could have led to the evolutionary development of adaptive feeding strategies of phagotrophs rather than to the development of mixotrophic feeding strategies, which would have implied major additional energetic costs. For phytoflagellates, the development of special adaptations for bacterial grazing in sediments might have been very costly in addition to the maintenance of the photosynthetic apparatus, since it is not clear which part of bacteria is actually freely available for flagellates in sediments (Dietrich & Arndt 2000).

## 6.2. System specific aspects

Sediments are characterized by steep vertical and horizontal gradients of light, oxygen, and other physical and chemical factors, which generate a very heterogeneous environment on very small spatial scales (<100μm, e.g. Kühl & Jørgensen 1994). Many of these gradients require special physiological adaptations of the organisms. In addition, the sediment surface layer along the shoreline is exposed to rapid environmental changes. Differences in the depth and turbidity of the overlying water and the corresponding light field, in the salinity and temperature all affect microbial community structure (Admiraal 1977, Pinckney & Zingmark 1991, Kühl & Jørgensen 1994).

Is was proposed and supported by experimental studies that temporarily or spatially heterogeneous environments with respect to resources should favor mixotrophs, whereas homogeneous conditions should favor strict autotrophs and heterotrophs over mixotrophs (Beaver & Crisman 1989, Berninger et al. 1986, Bird & Kalff 1987, Holen & Boraas 1995). With regard to communities, Jones (1994) suggested mixotrophy to be an adaptive strategy, providing greater flexibility in the planktonic environment, which is prone to unpredictable

spatial and temporal fluctuations. In the present study, mixotrophy was assumed to play a pivotal role in food web dynamics in heterogeneous environments like sediments and sea ice.

These implications proved to be true for sea ice that was investigated beside sediment and coastal plankton. Sea ice is characterized by strong seasonal and spatial variability in light availability, temperature, porosity, brine salinity and availability of inorganic nutrients (Weeks & Ackley 1982, Maykut 1985, Gleitz et al. 1995). In the present study, highest mixotrophic contributions were found in the brine of sea ice, although they were not as high as in some of the previous plankton studies (e.g. Sanders et al. 2000, Havskum & Riemann 1996, Arenovski et a. 1995, Safi & Hall 1999). Sea ice can be an effective barrier to light transmission, and sea ice algae have to be physiologically adapted to living at low light conditions (Thomas & Diekmann 2002). Palmisano & Garrison (1993) proposed sea ice algae to be able to switch from autotrophy to heterotrophic uptake of organic matter during periods of very low light, especially in winter. Stoecker et al. (1998) even reported considerable numbers of mixotrophic dinoflagellates from land-fast sea ice in the Antarctic, appearing in certain times of the year. Apparently, a number of different survival strategies have evolved in heterogeneous sea ice habitats, and mixotrophy was shown to be one of these strategies. However, only one experiment with sea ice was conducted, presenting a "snapshot" of microbial dynamics in the brine channels of sea ice in one particular floe at one particular point of time. Therefore, more experimental field work is required to further evaluate mixotrophic feeding strategies in sea ice.

If mixotrophy is an advantageous strategy in heterogeneous environments such as sea ice, it is surprising that mixotrophic feeding strategies were not found to play a major role in the sediments investigated in this study. Mixotrophy in sea ice might be an essential survival strategy for many organisms, which become trapped in the brine channels and cannot escape from unfavorable environmental conditions, limiting phagotrophy or photosynthesis. In water columns with a great mixing depth, the organisms can also not escape from changing environmental conditions, such as decreasing light intensities, when they are carried into deeper water layers. Microorganisms can freely move on small spatial scales relative to their body size, but can not ascend or descend in the water column on scales of up to 100m, when environmental conditions change. When plankton organisms are carried into deeper water layers, they have to cope with potentially unfavorable conditions and are dependent on being carried again to surface waters. In contrast, organisms in sediments can freely move along the vertical gradients on small spatial scales of millimeters to centimeters; for instance, they can ascent towards better light conditions when light becomes limiting in deeper sediment layers.

Given these implications, mixotrophic feeding strategies might be essential for survival in pelagic environments as well as in brine channels of sea ice, whereas in coastal sediments, they play a different role for the organisms.

Characteristics of bacterial prey can be another deciding factor in influencing mixotrophic feeding strategies. In sediments, a significant amount of bacteria is associated with particles; estimates range from 50-99% (Weisse & Rheinheimer 1978, Sich 1990). Bacteria are attached to sand grains with polymer strands (Weisse & Rheinheimer 1978); alternatively they are found in protected environments formed by quartz crystalline structures and within detritus. Starink et al. (1996b) proposed that bacteria within micro refuges are not only protected from mechanical stress as pointed out by Weisse and Rheinheimer (1978), but probably also from predation by protozoa. Due to the great variety of interstitial and attached bacteria, phagotrophic protist populations living in such environments were proposed to have a wide range of feeding strategies in order to enhance niche segregation (Starink et al. 1994b). Patterson et al. (1989) reviewed some aspects of feeding behavior of benthic protists and found a variety of specialized organelle structures for feeding. Feeding preferences of heterotrophic microflagellates were demonstrated by Caron (1987) and Sibbald & Albright (1988) under laboratory conditions. Starink et al. (1994b) found higher experimental food particle uptake rates using labeled sediment particles compared to monodispersed tracers and suggested that a significant number of protists in sediments might be specialized in removing bacteria from particles. Great niche segregation for bacterial grazers, high bacterial abundances and rarely limiting nutrient conditions might explain the lower importance of mixotrophy in coastal sediments (see 6.1. Evolutionary aspects).

In some definitions of mixotrophy, osmotrophy by algae is included (Jones 1994, Lewitus & Kana 1995, Raven 1997 and Riemann et al. 1995), whereas in the present study only phagotrophy was considered as heterotrophic nutritional mode. Many phytoplankters can take up dissolved organic carbon (Lewitus & Kana 1995, Raven 1997 and Schnepf & Elbrächter 1992), or under inorganic nutrient stress, use dissolved amino acids or other organic sources of nitrogen (Michaels 1988). Certain photosynthetic flagellates such as some species of *Euglena* are well known to be facultatively osmotrophic and are able to switch in darkness from photosynthetic carbon fixation to heterotrophic utilization of acetate and alcohol (Nisbet 1984). Tulonen et al. (1992) reported that the growth of some phytoflagellates in low light is stimulated in water rich in humic substances, possibly due to heterotrophic utilization of some molecular weight fractions of the humic material. Also in aggregates of seawater, other kinds of food apart from bacteria like macromolecules and dissolved organic matter were proposed

to serve as nutrition for phagotrophic protists (Tranvik et al. 1993). Since macromolecules and dissolved organic substances can be very abundant in sediments in contrast to many pelagic environments, it can be speculated that osmotrophy is a more advantageous strategy for phytoflagellates to support their autotrophic nutrition compared to phagotrophy and plays a more important role in benthic mixotrophic protists. Benthic diatoms, found to be able to use dissolved free amino acids or organic material like yeast extracts or glucose, were partly mixotroph under light limitation (Admiraal & Peletier 1979, Admiraal et al. 1984, Admiraal et al. 1987) support this hypothesis. However, further experimental investigation of mixotrophic feeding strategies in sediments, also including deeper sediments, is required in order to estimate their potential significance in this environment.

## 6.3. Food web consequences

Mixotrophic feeding strategies present a continuous spectrum ranging from almost pure heterotrophy to almost pure autotrophy (Sanders 1991). Some species act on one particular point of this range, others are able to pursue different nutritional modes and move along this spectrum. This results in a range of the relative importance of photosynthesis and phagotrophy, which is species specific and dependent on environmental factors such as light, nutrients etc. (Sanders 1991, Stoecker 1998, Jones 2000). Information on the physiological ecology of mixotrophic protists is crucial to understand their ecological role in food webs and their impact on trophodynamics and food web structure (Jones 1997, Jones 1994, Reimann et al. 1995, Stoecker 1991, Stoecker & Michaels 1991, Turner & Roff 1993). However, experimental data on the functional relationships of phototrophy and phagotrophy to availability of particulate food, light, and dissolved inorganic or organic nutrients are not available for most mixotrophic species. The physiological ecology of only a few planktonic mixotrophs has been investigated in detail (see references in Stoecker 1998). The gradient that was found in mixotrophic species ranging from heterotrophy to autotrophy supported that mixotrophy plays different roles and is regulated in different ways in different protists (Jones 1997, Jones 1994). Stoecker (1998) attempted to categorize mixotrophic strategies within the great diversity of mixotrophic protists in plankton. This was done in order to incorporate them into general food web models for aquatic ecosystems and thus to explore the impact of mixotrophy on ecosystem dynamics. Mixotrophy was categorized into six possible physiological types, with three different basic model types. One was the "ideal" mixotroph, where phagotrophy and phototrophy are balanced. The second one was a phagotrophic algae,

being primarily phototrophic, feeding when a) DIN is limiting, b) when a trace organic growth factor is limiting or c) when light (carbon) is limiting. And the third one was a photosynthetic "protozoa", being primarily phagotrophic and photosynthesizing when a) prey is limiting or b) when supplementary carbon nutrition is required. These different functional types of mixotrophy should affect the total productivity of the microbial food web in different ways. Phagotrophy by algae may decrease the total microbial food web production by increasing consumption and respiration of fixed carbon within the microbial food web, whereas photosynthesis by protozoa may increase total production due to increased photosynthesis and more efficient usage of ingested nutrients (C, N, P and perhaps Fe). The effect of mixotrophy on trophic transfer from the microbial food web to metazoa is difficult to predict (Holen & Boraas 1995); it should depend on the type of mixotrophy. When mixotrophy enhances for instance the total production within the microbial food web, it should also enhance trophic transfer to metazoa. The trophic transfer is also likely to depend on the relative quality/suitability of the mixotroph and its prey as food for metazoa. For example, consumption of bacteria by mixotrophic flagellates, which are ingested by many metazoa, may make a fraction of bacterial biomass available to higher trophic levels. In contrast, the consumption of small ciliates, which are high quality food for metazoa, by dinoflagellates, that are similar or poorer quality food for Metazoa, may decrease trophic transfer from the microbial food web to higher trophic levels (Stoecker 1998).

Mixotrophy also influences food web structure and function by affecting competition among phytoplankton or heterotrophs (Rothhaupt 1996a, Stoecker et al. 1997, Thingstad et al. 1996). For instance, the mixotrophic chrysophyte *Ochromonas* sp. was grown together with the obligate phagotroph *Bodo* in batch culture in the dark, where the obligate phagotroph outgrew the mixotroph. However, when grown together in the light, the mixotroph eventually outcompeted the phagotroph once the bacterial prey had been grazed down to a density that limited the growth of the phagotroph (Rothhaupt 1996ab). Controlled experimental tests of competition between mixotrophic protists and heterotrophs or autotrophs are rare and more work of this kind would be very valuable (Jones 2000).

These examples demonstrated that the type of mixotrophy is important for food web implications. But also the relative balance between different carbon and energy sources in the system can be of decidal importance, determining the ecological role of mixotrophs. For example, a clearwater lake with high light availability and low dissolved organic carbon should differ from humic lakes with low light availability but high DOC. Conditions of favorable light and low DOC offer little advantage to mixotrophs, so that most carbon and

phosphorus flux should be through obligate phototrophs to larger zooplankton, with a smaller flux through bacteria and obligate heterotrophs (Jones 2000). Conversely, conditions of unfavorable light and high DOC should disadvantage obligate phototrophs, because of both light limitation and increased competition with bacteria for inorganic P. Mixotrophy should be attractive then, because it allows for supplementation of photosynthetically fixed carbon with that from ingested bacteria as well as proving an alternative source of essential nutrients from ingested bacteria (Jones 2000). As a consequence, mixotrophs become an important link in the flux of both carbon and phosphorus through the plankton community. Most examples illustrating the potential ecological impact of mixotrophs on food web dynamics are derived from plankton communities. Similar implications can be found in benthic environments and further research on mixotrophic feeding strategies, including osmotrophy, is required in order to fully understand microbial food web dynamics in sediments.

## 6.4. Regulation and complex response

Field studies and experimental data have shown that it is very difficult to predict where and when mixotrophy will be an important ecosystem property. This is due to the lack of knowledge of physiological properties of most mixotrophic protists and to their extreme variability in temporal and spatial scales. This variability is dependent on an array of environmental factors such as light, nutrients, prey availability, DOC etc. These factors influence mixotrophs within microbial food webs on three different levels; on the cellular level of an individual organism, on population level and on community level, which in turn affect microbial food web dynamics (Fig. 6.1.). For instance, nutrient conditions affect mixotrophs on a cellular level, influencing the balance between phagotrophy and photosynthesis and their functional response. Nutrient conditions also affect the balance between phagotrophy and photosynthesis on the population level as well as numerical response of the population. On a community level, nutrient conditions may affect the competition of mixotrophs and obligate autotrophs, the feeding impact on autotrophs and bacteria as well as the grazing impact of predators. Different factors act on all three organisational levels in different ways, and also on different time scales, which hamper the prediction of the ecological role mixotrophy might perform in natural communities, either in pelagic or in benthic ones.

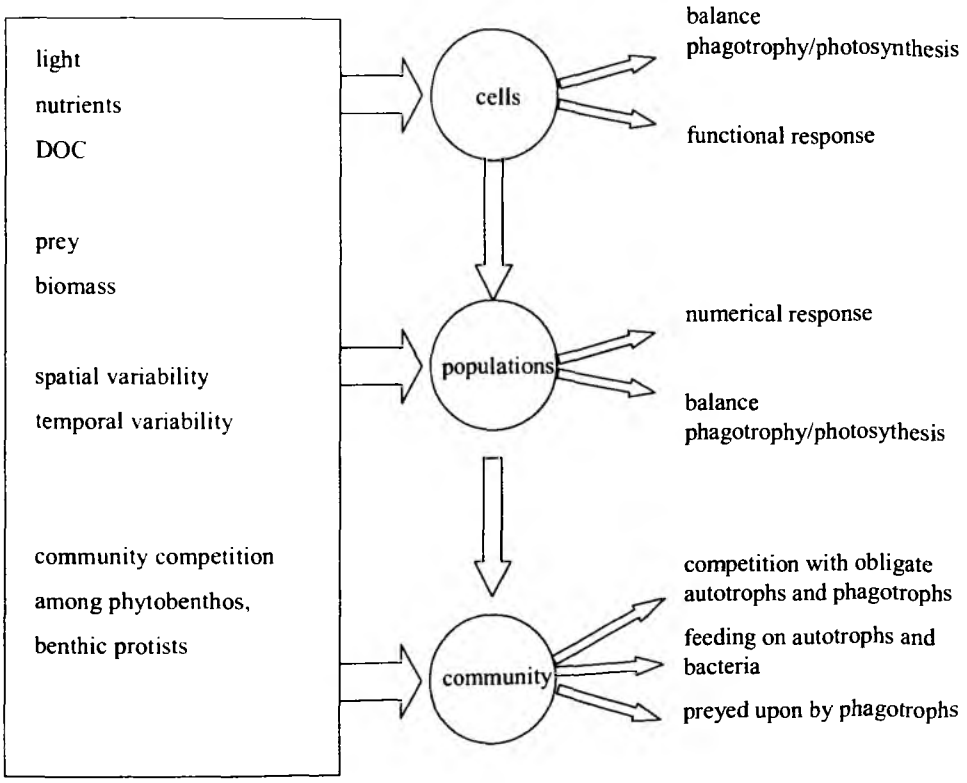



**Fig. 6.1.** Environmental biotic and abiotic factors affecting mixotrophy on three organizational levels (cell – population – community), which in turn influence food web dynamics.

Although the present study indicated a minor importance of mixotrophic flagellates in coastal sediments, more experimental fieldwork is required to support these findings. The experiments provide valuable information about the potential significance of MNF in coastal sediments, but at the same time present only "snapshots" of microbial community dynamics at different sites and different points of time. The situation could change under different environmental conditions; therefore it would be helpful to investigate seasonal variations of particular microbial sediment communities with regard to mixotrophy on a finer temporal resolution than it was possible here. To fully understand the link between the microbial food web and metazoa, it is also necessary to include predators of mixotrophic organisms into studies, for it could be possible that high numbers of mixotrophs are active, but cannot be detected because they are grazed to great extents.

One major problem of tracer experiments, which are necessary for the investigation of mixotrophs in either sediments or plankton community, is the "black box" character of the study. Fixed flagellates, which are investigated with an epifluorescence microscope, cannot be

taxonomically determined. It is only possible to group them to trophic levels (heterotroph, autotroph or mixotroph), but rarely to particular taxonomic groups. On the other hand, protists investigated alive or by electron microscopy, cannot be identified as mixotrophs due to the lack of experimental evidence of feeding in addition to the possession of plastids. Therefore, it is impossible until now to investigate the quantitative and qualitative (on species level) importance of mixotrophs at the same time. It would be of invaluable importance to combine the two different approaches of investigating microbial community structure in the field by determining both quantitative relations of different entities and microbial community composition at species level, i.e. to know which species represent particular entities like mixotrophs. In combination with laboratory studies of particular mixotrophic species, it would finally be possible to incorporate them into trophic models and to estimate their role in nature.

# Chapter 7

## Summary – Zusammenfassung

### Summary

Mixotrophy presents the ability of an organism to combine autotrophic and heterotrophic modes of nutrition and is a common phenomenon in aquatic food webs. Acting on more than one trophic level, mixotrophy adds complexity to original models for the microbial loop, which is an important pathway for carbon and nutrient flux in aquatic ecosystems. Mixotrophic feeding strategies, presenting a spectrum of almost pure autotrophy to almost pure heterotrophy, are species specific and dependent on environmental factors, such as light, nutrient conditions, prey abundances etc. Numerous planktonic studies described mixotrophic occurrence, their physiological peculiarities, their contribution to nutrient recycling and their function within microbial food webs. It was shown that mixotrophic occurrence and abundances in marine and freshwater ecosystems are highly variable in temporal and spatial scales, but that mixotrophs can play a major role as primary producers and as phagotrophs.

In contrast to the plankton, the ecological role of benthic protists is poorly investigated such as the occurrence or the ecological role of benthic mixotrophs. Sediments are characterized by steep vertical and sometimes horizontal gradients of light, oxygen, nutrient concentrations and other physical and chemical factors that generate a pronounced heterogeneity. Mixotrophy was proposed to be an advantageous strategy in this heterogeneous environment due to the ability to respond to changing environmental factors with a switch of the trophic mode.

In the present study mixotrophic nanoflagellates in coastal marine sediments were investigated. Tracer experiments using surrogate food particles were conducted to identify mixotrophic flagellates by the presence of cellular plastids and ingested tracer particles.

A tracer method using fluorescently labeled bacteria (FLB) was modified for the systems investigated, using monodispersed FLB that were isolated just before the experiments out of the predator's natural habitat (experimental sampling site). In the following, the quantitative importance and the ecological role of mixotrophs were investigated in dependence of light and nutrient conditions and along small-scale vertical and horizontal gradients at the main sampling site at Falckenstein Beach in the Western Baltic Sea. Mixotrophs showed varying abundances and contributions to the flagellate community, which could not be attributed to

particular environmental factors in all cases. Mixotrophs responded to light and nutrient limitation with increased phagotrophic activity in a set of experiments conducted in February, but not in October. Along vertical gradients, mixotrophs showed increasing phagotrophic activity with decreasing light intensities on one type of sediment, but not in a finer grained sediment. These disparate responses in temporal and spatial scales were attributed to differences in community composition of mixotrophs and in the relative importance of environmental factors that determine mixotrophic feeding strategies. These results supported the fact that mixotrophy is an extremely variable phenomenon, which is difficult to be attributed to particular factors. Overall, mixotrophs contributed low portions to the total nanoflagellates (max. 4%) at Falckenstein Beach, contributing higher portions to the total phytoflagellates (10-15%) than to total bacterivores (2-5%).

Energetical costs for mixotrophy were assumed to be too high for organisms in the low saline Western Baltic Sea due to physiological constraints caused by osmotic stress. Therefore the significance of mixotrophs was investigated along a salinity gradient in 5 different systems in sediments and the overlying water column in Northern Germany, in 3 fully marine systems in the Pacific Ocean in Southern California and in sea ice and plankton from the Greenland Sea. Sea ice is characterized by strong seasonal and spatial variability in light availability, temperature, porosity, brine salinity and availability of inorganic nutrients and was therefore also proposed to favor mixotrophic feeding strategies. In the sediment and plankton communities, mixotrophs contributed maximum portions of 7% to the total nanoflagellates, which were only exceeded in the brine of sea ice. Mixotrophic contributions increased with increasing salinity, supporting the initial hypothesis. But even in fully marine sediments, mixotrophic nanoflagellates contributed maximum portions of 25% to the total PNF and 5-10% to the total grazers, having a considerable potential as primary producers but playing a minor role as bacterivores. Highest contributions of mixotrophs in brine indicated their potential importance in sea ice. Mixotrophic feeding strategies were proposed to play a greater role in oceanic plankton and sea ice than in coastal sediments. Organisms cannot escape unfavorable conditions in brine channels or water columns with a great mixing depth, when carried into deeper water layers, whereas in sediments the organisms can feely move along the small-scale gradients. Furthermore, it was proposed that flagellates have rather evolved adaptive feeding strategies to graze on the great variety of attached and interstitial bacteria than having evolved mixotrophic feeding strategies, which are less required in coastal sediments due to rarely limiting nutrient conditions or prey abundances. In a wider sense of

mixotrophy, osmotrophy of benthic microalgae was assumed to have a greater importance in sediments than phagotrophic feeding strategies in phytoflagellates.

## Zusammenfassung

Mixotrophie ist die Fähigkeit eines Organismus, heterotrophe und autotrophe Ernährungsmodi in sich zu vereinen und ist ein weit verbreitetes Phänomen in aquatischen Nahrungsnetzen. Durch das Wirken mixotropher Organismen auf mehr als einer trophischen Ebene gewannen ursprünglich vorgeschlagene Modelle für die mikrobielle Nahrungsschleife an Komplexität, die einen wichtigen Weg für den Kohlenstoff- und Nährstofffuß in aquatischen Ökosystemen darstellen. Mixotrophe Nahrungsstrategien stellen einen Gradienten dar, der fast von purer Autotrophie zu purer Heterotrophie reicht. Dieser Gradient ist artenspezifisch und hängt von Umweltfaktoren wie Licht, Nährstoffkonzentrationen und Beuteabundanzen ab. In einer Vielzahl von Planktonstudien wurde das Auftreten von Mixotrophen, ihre physiologischen Besonderheiten, ihr Beitrag zur Nährstoffregeneration und ihre Funktion innerhalb des mikrobiellen Nahrungsnetzes beschrieben. Es zeigte sich, dass das Auftreten von Mixotrophen und ihre Abundanzen in marinen und limnischen Ökosystemen zeitlich und räumlich extrem variabel ist, dass Mixotrophe aber eine große Rolle als Primärproduzenten und Phagotrophe spielen können.

Im Gegensatz zum Plankton ist die ökologische Rolle benthischer Protisten kaum erfasst wie z.B. das Auftreten und die ökologische Rolle benthischer Mixotropher. Sedimente sind durch steile Vertikal- und Horizontalgradienten von Licht, Sauerstoff, und anderen physikalischen und chemischen Faktoren charakterisiert und erzeugen so eine ausgeprägte Heterogenität. Aufgrund der Fähigkeit mixotropher Organismen auf wechselnde Umweltbedingungen mit einem Wechsel des Ernährungsmodus zu reagieren, wurde angenommen, dass Mixotrophie eine vorteilhafte Ernährungsstrategie in Sedimenten sein kann.

In der vorliegenden Studie wurden mixotrophe Nanoflagellaten in küstennahen marinen Sedimenten untersucht. Sogenannte "tracer"-Experimente wurden mit markierten Nahrungspartikeln durchgeführt, um mixotrophe Flagellaten anhand ihrer Chloroplasten und ingestierten Nahrungspartikeln zu erkennen.

Eine "tracer"-Methode mit fluoreszierenden markierten Bakterien (fluorescently labeled bacteria, FLB) wurde für die untersuchten Systeme modifiziert, indem Bakterien zur FLB Präparation direkt vor dem Experiment aus dem natürlichen Habitat des Räubers isoliert

wurden (aus der experimentellen Probenahmestelle). Im Folgenden wurde die quantitative und ökologische Rolle mixotropher in Abhängigkeit von Licht- und Temperaturbedingungen untersucht, außerdem entlang eines kleinskaligen Vertikal- und Horizontalgradienten an der Hauptprobennahmestelle am Falckensteiner Strand in der Westlichen Ostsee.

Mixotrophe zeigten variable Abundanzen und Anteile an der Flagellatengemeinschaft, die nicht in allen Fällen bestimmten Umweltbedingungen zugeordnet werden konnten. Mixotrophe reagierten auf Licht- und Nährstofflimitation mit ansteigender phagotropher Aktivität in zwei Experimenten, die im Februar durchgeführt wurden, wohingegen sie in einem Experiment im Oktober gar nicht reagierten. Entlang der vertikalen Gradienten zeigten Mixotrophe ansteigende phagotrophe Aktivität mit abnehmender Lichtintensität in einem bestimmten Sedimenttypen, nicht jedoch in einem feineren Sediment. Diese räumlich und zeitlich verschiedenen Reaktionen wurden auf Unterschiede in der Gemeinschaftszusammensetzung zurückgeführt wie auch auf die unterschiedlichen Faktoren, die die relative Bedeutung mixotropher Nahrungsstrategien bestimmen. Diese Ergebnisse unterstützen die Tatsache, dass Mixotrophie ein extrem variables Phänomen ist, welches nicht einfach auf bestimmte Faktoren zurückgeführt werden kann. Insgesamt trugen Mixotrophe kleine Anteile zu den Gesamtnanoflagellaten bei am Falckensteiner Strand bei (max. 4%), mit größeren Anteilen an den Gesamtphytoflagellaten (10-15%) als an den gesamten Bakterivoren (2-5%).

In der Westlichen Ostsee wurden energetische Kosten für Mixotrophie sehr hoch eingeschätzt aufgrund von osmotischem Stress durch einen niedrigen Salzgehalt. Daher wurde die Bedeutung mixotropher Nanoflagellaten entlang eines Salinitätsgradienten in 5 verschiedenen Sediment-Systemen und der darüberliegenden Wassersäule untersucht, außerdem an 3 vollmarinen Standorten im Pazifik in Südkalifornien und im Meereis und Plankton in der Grönlandsee. Meereis ist durch eine saisonal und räumlich stark variierende Lichtverhältnisse, Temperaturen, Porosität, Solesalinität und Verfügbarkeit anorganischer Nährstoffe charakterisiert, was mixotrophe Ernährungsstrategien begünstigen könnte.

Im Sediment und im Plankton wurden maximale Anteile von 7% der Mixotrophen an Gesamtnanoflagellaten gefunden, die nur in der Sole des Meereises höher waren. Mixotrophe Anteile stiegen mit ansteigender Salinität, was die ursprüngliche Hypothese bestätigte. Doch auch in vollmarinen Sedimenten machten Mixotrophe maximale Anteile von 25% an den Gesamtphytoflagellaten aus und 5-10% an den gesamten bakterivoren Flagellaten, wodurch sie ein gewisses Potential als Primärproduzenten hatten, jedoch eine kleine Rolle als Bakterivore spielten. Die höchsten Anteile der Mixotrophen wurde in der Sole des Meereises

gefunden, was ihre potentielle Bedeutung im Meereis unterstreicht. Es wurde angenommen, dass mixotrophe Nahrungsstrategien eine größere Rolle im ozeanischen Plankton und im Meereis spielen als in küstennahen Sedimenten. In den Solekanälen sind die Organismen nicht in der Lage ungünstigen Umgebungsbedingungen zu entfliehen, was auch der Fall in Wassersäulen mit einer großen Durchmischungstiefe ist, wenn die Organismen in tiefere Wasserschichten getragen werden; in Sedimenten dagegen können sich die Organismen frei entlang der kleinskaligen Gradienten bewegen. Desweiteren wurde angenommen, dass Flagellaten im Laufe der Evolution vielleicht eher adaptive Nahrungsstrategien für das Abweiden der diversen angehefteten und interstitialen Bakterien entwickelt haben als mixotrophe Nahrungsstrategien, da diese im küstennahen Sediment durch selten limitierende Nährstoff- oder Beutekonzentrationen weniger benötigt werden. Im weiteren Sinne von Mixotrophie wurde angenommen, dass Osmotrophie als mixotrophe Ernährungsstrategie eine größere Rolle für Phytoflagellaten in Sedimenten spielt als Phagotrohpie.

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# Abbreviations

## Experiments

| | |
|---|---|
| CH 2: Exp. I ($FLB_{nat/sed}$) | Experiment I: monodispersed natural benthic FLB |
| CH 2: Exp. II ($FLB_{nat/sed}$) | Experiment II: monodispersed natural benthic FLB |
| CH 2: Exp. III (FLS) | Experiment III: fluorescently labeled sediment versus monodispersed natural benthic FLB |
| CH 2: Exp. IV ($FLB_{Hal}$) | Experiment IV: FLB made of the bacterial culture strain *Halomonas halodurans* versus |
| $FLB_{nat\ plank}$ | monodispersed natural planktonic FLB |
| CH 5: $Exp.I_{NB}$ | Experiment I: Newport Beach |
| CH 5: $Exp.II_{HB}$ | Experiment II: Huntington Beach |
| CH 5: $Exp.III_{Cat}$ | Experiment III: Catalina Island |
| CATsand | sandy sediment on Catalina Island |
| CATmud | muddy sediment on Catalina Island |
| CH 5: $Exp.IV_{Falck}$ | Experiment IV: Falckenstein Beach |
| FALCKshallow | shallow sediment samples (0.5m depth) |
| FALCKdeep | deep sediment samples (2.5m depth) |
| CH 5: $Exp.V_{Laboe}$ | Experiment V: Laboe |
| CH 5: $Exp.VI_{Poel}$ | Experiment VI: Poel |
| CH 5: $Exp.VII_{Lake}$ | Experiment VII: Schöhsee |
| CH 5: $Exp.VIII_{Sylt}$ | Experiment VIII: Sylt |
| SYLT lt | sediment samples at low tide |
| SYLT ht | sediment samples at high tide |
| CH 5: $ExpIX_{Ice}$ | Experiment IX: Greenland Sea |

## Abbreviations used in the Text

| | |
|---|---|
| ANOVA | analysis of variances |
| DAPI | 4', 6-Diamidino-2-phenylindol |
| df | degree of freedom |

| | |
|---|---|
| DTAF | 5-(4,6-dichlorotriazin-2-yl)aminofluorescein |
| FLB | fluorescently labeled bacteria |
| FLS | fluorescently labeled sediment |
| HNF | Heterotrophic nanoflagellates |
| MNF | Mixotrophic nanoflagellates |
| PNF | Phototrophic nanoflagellates |
| SSW | sterile filtered sea water |
| TSB | trypticase soy broth |

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